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Exploring legume protein derivatives for enhanced essential element bioaccessibility

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Exploring legume protein derivatives for enhanced essential element bioaccessibility

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Abstract

Essential element dyshomeostasis is a tenacious nutritional risk factor underlying the pathophysiology of numerous diseases in humans. A fundamental cause is the inadequacy of elemental bioaccessibility from dietary sources, especially plant-based foods. Out of the essential dietary elements, iron (Fe) and zinc (Zn) are among the broader class of micronutrients whose deficiencies affect approximately one-third of people worldwide. Oral supplementation is the first line of therapy being both accessible and affordable. However, conventional elemental salt supplements often create an imbalance between low bioaccessibility and toxic overload within the body, leading to adverse side effects and unknown safety with cumulative exposure. Some food-derived biomolecules possess physicochemical properties that may be utilised to enhance elements' solubility, and hence bioaccessibility during digestion for improved absorption. As such, the current investigation explored the potential properties of legume-derived protein products that may enhance the bioaccessibility of Fe and Zn.

The study began by extracting water- and NaCl-soluble protein fractions from three Australian-grown pulses (pea, lupin and faba), with their effects on fortified Fe and Zn bioaccessibility examined using an *in vitro* simulated gastrointestinal model. Both promotive and inhibitory effects were observed depending on the fraction and protein source, as well as the digestion state. Lupin and faba salt extracts enhanced Fe(II) and Zn small intestinal solubility under fed and fasted digestion states, respectively. Meanwhile,

water-soluble pea proteins showed the greatest potential to solubilise Fe(III), mostly during gastric digestion.

Subsequent to the preliminary study, pea protein extract was selected to be subjected to bioprocessing approaches involving either enzymatic hydrolysis (protease, phytase, or both) or inoculated fermentation (*Lactobacillus plantarum*) to enhance its potential as carrier of Fe(III) or Zn during small intestinal digestion. All treatments involving phytase were effective at enhancing Fe(III) solubility during small intestinal digestion, whereas all treatments involving protease were able to enhance Zn solubility only under the fed state of digestion. This reflected differences in the binding behaviour between Fe(III) and Zn with the proteins during digestion, indicating that they be investigated separately.

Focusing on iron as the most commonly deficient micronutrient, the third study characterised the properties of the soluble Fe(III)-binding peptides potentially responsible for the enhanced solubility observed in the previous chapters. The unpurified hydrolysate was found to bind 5.3 mg of soluble iron/g of powder. The Fe(III)-binding peptides that were separated using IMAC-Fe(III) and characterised using LC-MS/MS were found to be rich in Glu, Asn, Lys and Leu, and low in sulfur-containing amino acids Met & Cys. Further bioinformatics analyses identified 15 novel peptides (< 1.5 kDa) that were suitable for enhancing Fe(III) bioaccessibility based on their abundance, chelation score, isoelectric point and hypo-allergenicity.

Although pea protein hydrolysates were able to enhance Fe(III) bioaccessibility in the previous studies, its poor overall bioaccessibility can lead to increased iron levels in the lower gut, underscoring a need to examine their effects on the gut microbiota. The final study investigated the effects of iron fortification with and without pea protein fractions (intact and hydrolysate) on both small and large intestinal bioaccessibility, using the INFOGEST gastrointestinal model with colonic fermentation using a human faecal culture. Following 24 h of fermentation, all treatments involving iron fortification saw a decline in within-species diversity, relative increases in members of *Proteobacteria* and a loss of *Lactobacilliaceae*. Fortification of the hydrolysate was found to reverse the intact protein's inhibitory effects on small intestinal iron solubility, but led to a decline in total short-chain fatty acids (SCFAs), and significant increases in *Proteobacteria* comparable to that found in the iron salt control. Meanwhile, the fortified intact protein saw an increase in SCFAs and in the butyrate-producing family *Propionibacteriaceae.*

This thesis addresses the critical role of legume protein fractions as modulators of fortified iron/zinc bioaccessibility, challenging ways to overcome their reputation as absorption inhibitors. It expands extant knowledge on how properties of legume peptides that bind to iron and/or zinc can be associated with promotive or inhibitory effects on iron/zinc bioaccessibility during digestion, as well as diverging effects on the gut microbiota. Outcomes of the study prompts that further research on ways of improving legume proteins' digestibility may assist to promote fortified Fe and Zn bioaccessibility.

Declaration

This is to certify that:

i. the thesis comprises only my original work towards the PhD except where indicated in the Preface,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signed: Yianna Y. Zhang

Date: 28/03/2023

Preface

This thesis is based on the following published journal papers for which I was the lead author:

1. Zhang, Y. Y., Stockmann, R., Ng, K., & Ajlouni, S. (2020). Revisiting phytate-element interactions: implications for iron, zinc and calcium bioavailability, with emphasis on legumes. *Critical Reviews in Food Science and Nutrition*, 1-17.

2. Zhang, Y. Y., Stockmann, R., Ng, K., & Ajlouni, S. (2021). Opportunities for plant-derived enhancers for iron, zinc, and calcium bioavailability: A review. *Comprehensive Reviews in Food Science and Food Safety*, *20*(1), 652-685.

3. Zhang, Y. Y., Stockmann, R., Ng, K., & Ajlouni, S. (2021). The role of legume peptides released during different digestion stages in modulating the bioaccessibility of exogenous iron and zinc: An *in-vitro* study. *Current Research in Food Science*, *4*, 737-745.

4. Zhang, Y. Y., Stockmann, R., Ng, K., & Ajlouni, S. (2022). Bioprocessing of Pea Protein can Enhance Fortified Fe but Reduce Zn *in vitro* Bioaccessibility. *Journal of Agricultural and Food Chemistry*, 70(4), 1241-1251.

5. Zhang, Y.Y., Broadbent, J.A., Stockmann, R., Ng, K., Suleria, H., Stockwell, S., Shaik, N.E.K., Unnithan, R.R., Ajlouni, S. (2022). Characterization of Fe(III)-binding peptides from pea protein hydrolysates targeting enhanced iron bioavailability. *Food Chemistry*, 405, 134887

6. Zhang, Y.Y., Stockmann, R., Ng, K., & Ajlouni, S. (2023). Hydrolysis of pea protein differentially modulates its effect on iron bioaccessibility, sulfur availability, composition and activity of gut microbial communities *in vitro*. 14(11), 5182-5195.

Contents from this thesis have also been presented at the following conferences:

1. Zhang, Y. Y., Stockmann, R., Ng, K., & Ajlouni, S. (11-12 October 2021). *Peptides formed during in vitro digestion of legume proteins have mixed effects on the bioaccessibility of fortified Fe and Zn salts* [Poster presentation]. AIFST21 Conference: Food Science - delivering in a changing world, online.

2. Zhang, Y. Y., Stockmann, R., Ng, K., & Ajlouni, S. (18-21 September 2022). *Enzymatic hydrolysis of pea proteins enhances fortified Fe bioaccessibility and positively modulates SCFA production during in vitro colonic fermentation* [Poster presentation]. 5th Food Structure and Functionality Symposium, Cork, Ireland.

I have contributed more than 50% to all of the above journal articles and conference presentations pertaining planning, execution, and writing. All manuscripts have been reformatted in correspondence with the thesis style proposed by the University of Melbourne. All of my contributions were conducted under the guidance of Dr. Regine Stockmann, Dr. Ken Ng and Associate Professor Said Ajlouni, who also played important roles in the ideation behind these studies, and provided editorial advice for various drafts of the manuscripts. Dr. James A. Broadbent, Dr. Sally Stockwell, Noor E. Karishma Shaik and Associate Professor Ranjith R. Unnithan have contributed to the execution of laboratory studies in the 5th manuscript, as well as reviewing the final draft. Dr. Hafiz Suleria assisted in the research approach and preliminary work behind the 5th manuscript and reviewed the final draft.

Some of the laboratory analyses involved in the study (ICP-OES, LECO) were performed with assistance from TrACEES (The Melbourne Trace Analysis for Chemical, Earth and Environmental Sciences). Dr. Augustine Doronila from the platform provided me with training in ICP-OES analyses.

The work conducted for this thesis has been supported by the Melbourne Research Scholarship at the University of Melbourne, and the Postgraduate Scholarship at the Commonwealth Scientific and Industrial Research Organisation (CSIRO).

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In the immortal words of Abraham Maslow, "…it is tempting, if the only tool you have is a hammer, to treat everything as if it were a nail". As such, the knowledge availed from this thesis is underpinned by collaborative efforts of well-informed individuals with varying expertise, united by a mission to understand a pervasive problem incumbent on us.

To my partner Ren Guo, thank you for being my lifeblood throughout this journey. Not only for always being there for wiping my tears, but also for gifting your capable hands to enliven our scientific work into literal forms of art.

I have been privileged to be under the tutelage of many, but particularly A/Prof. Said Ajlouni, Dr. Ken Ng, and Dr. Regine Stockmann, whose patience and tolerance I will always be grateful for;

Said, from the moment you stayed after class in 2016 to explain that it was okay that my first ELISA experiment failed, I have only wanted to make you proud. This study could not have been possible without the power of your kindness and generosity.

Ken, thank you for embodying to me the humble art of disagreeing without being disagreeable. I will never forget that you wished me happiness whilst I was in an abyss of despair, and also the importance of language clarity in distilling technical information.

Regine, I am constantly in awe of your ardent drive, as well as immense capacity in leadership and networking whilst dutifully juggling multiple life roles. Your skills have been critical to the execution of this study, bridging us amongst many colleagues.

I have been fortunate to have Prof. Robyn Warner and Dr. Hafiz Suleria as members of my PhD committee, who have generously made time to accommodate my milestones, in addition to some invaluable advice.

I am appreciative of my wonderful colleagues at both the university and CSIRO for their emotional and technical support, including MD Nur Houssain, Siyao Liu, Fasiha Zahid, Zhenzhao Li, Anna Wittwer, Yit Tao Loo, Yipeng Chen, Wenan Cai, Xinwei Ruan, Meng Tian He, Aniket More, Wei Zou and Adil Malik. Additional thanks are given to Dr. James Broadbent, Dr. Sally Stockwell, Noor E. Karishma Shaik, A/Prof Ranjith Unnithan, and the team at TrACEES for collaborating with us in some of the experiments. I would also like to recognise the exemplary laboratory officers & research office team within the School of Agriculture & Food for their exceptional organisation and reliable support.

Back to where the story first began, I am grateful for Mrs. Jackie Knight, the teacher who first informed me of the threats posed by dietary micronutrient deficiencies to human development. Thank you for planting a seed within the heart of an 18-year-old that became prefatory to this thesis. Thanks also to Ms. Julia Cafiso, my food technology teacher who believed in me from the beginning, but knew me well enough to tell others to not tell me, as to keep my ego in check.

To dad, you'll always be the best chemist in the world in my eyes.

To mum, thank you for all your love, and for showing me to fight for what we love.

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Chapter 1. General Introduction

Several chemical elements are collectively indispensable to human health serving a range of specific biological functions. These includes elements that (i) constitute structural components such as Ca, P and Mg in bones, (ii) are essential components (e.g. Fe, Zn and Cu) in metalloproteins and metalloenzymes, (iii) are involved in intracellular signalling messengers (e.g. Ca and P), (iv) facilitate redox state and acid-base balance (e.g. Na, K, Ca, Se), and (v) partake in osmoregulation (e.g. K, P and Na) (Replogle, Fleet, & Salt, 2011). Deficiencies of essential elements is a serious health issue that affected up to a third of the world's population, which is largely intake-related (Jariwalla, Niedwiecki, & Rath, 2010). Individuals affected by prolonged essential element deficiencies bear the burdens of various developmental, cognitive, and metabolic impairments; leading to premature mortality or severed quality of life (Gupta & Gupta, 2014). This impels significant aggregated social and economic costs scaling from households to nations, particularly for developing countries where over 98% of all malnourished people reside (Garg et al., 2018), many of whom are suffering from intergenerational malnutrition (Troesch et al., 2015).

Global malnutrition remains an ongoing dilemma, with 29.3% of the world moderately or severely food insecure in 2021 (FAO, IFAD, UNICEF, WFP, & WHO, 2022). This implies obstacles in adequate dietary acquisition of essential elements, which may be exacerbated in the future, as concentrations of essential elements in common edible crops tend to decline under projected climate change conditions (Dietterich et al., 2015; Loladze, 2014). Under such context, oral administration of essential elements through

supplementation or food fortification are amongst the most accessible approaches to reverse poor nutritional status. Intervention by supplements can mitigate insufficient dietary intake, but it can be a source of several technical problems. One key problem is that the amount of an given element absorbed by the human body does not necessarily increase linearly with the dose ingested (Nosratpour & Jafari, 2019). In order to interact with cells and tissues, these elements need to be in an ionised state for them to be released into water, the medium that comprises 60% of our body. However, most dietary elements are rooted in compound forms; ionically or covalently bound to other elements. Following ingestion, these compounds would have to be adequately dissolved at the site/s of absorption for the elements to be accessible. This process is termed bioaccessibility, which is the prerequisite for bioavailability – the total fraction of minerals in a given food or diet that is utilised by the body for physiological function, relative to the ingested amount (Guo et al., 2014; La Frano, de Moura, Boy, Lönnerdal, & Burri, 2014).

The concept of bioavailability is central to the development of cogent fortification and supplementation strategies, as elements are absorbed correspondingly to dosage, chemical form, delivery matrix, and host dynamics. Conventional supplements are composed of mineral salts, which often leads to poorly controlled pharmacodynamics and bioavailability for some elements. For example, common iron salts are reported to have low bioavailability at its major absorption site leading to constipation and excretion in stool (Tolkien, Stecher, Mander, Pereira, & Powell, 2015). However, higher doses have been linked to gastrointestinal side effects and toxicity from overload (Auerbach, 2022). Since dissociation of the mineral salt complex is required for bioaccessibility, the elements

ionised before and/or during digestion are prone to interactions with counterions from food or digestive fluids. This can produce adverse sensory problems and reduce elemental bioaccessibility (Sun et al., 2020), particularly in the context of monotonous plant-based diets where absorption inhibitors are common (Samtiya, Aluko, Puniya, & Dhewa, 2021).

In the search for alternatives with superior bioavailability and safety, food-derived nutraceuticals have been gaining increasing traction as desirable substitutes to traditional supplements (Guo et al., 2014). Some isolated food polymer fractions possess physicochemical properties that modulate nutrient absorption, such as binding, bulking and fermentation, solubility, water-holding capacity, viscosity and gel formation (Baye, Guyot, & Mouquet-Rivier, 2017; Belitz, Grosch, & Schieberle, 2009). These food fractions may be utilised to target nutrient release during small intestinal digestion, the primary absorption site for most elements. In particular, novel protein-derived fractions that may enhance mineral bioavailability have been characterised in a diverse number of food sources, such as calcium- and iron-binding peptides from milk (Cross, Huq, & Reynolds, 2007), soybean (Lv, Bao, Liu, Ren, & Guo, 2013) and shrimp (Huang, Ren, & Jiang, 2011). Most of these have been animal-based products, with plant-derived sources increasingly considered but not extensively explored (Eckert et al., 2016). One barrier is that whilst plant metabolites such as peptides and polyphenols possess metal-binding properties, the binding can inhibit elemental bioaccessibility due to their intrinsically low digestibility. Given the potential disparity between plant metabolomes, the fractions of interest and/or degree of post-harvest processing, this represents an untraversed gap in the understanding the potential of plant-based products to enhance elemental bioaccessibility.

By virtue of their sustainable production, legume seeds or pulses are key targets of nutraceutical products as a parent material (Rizzello et al., 2016). The safety and availability of pulses are well reigned, as a primary source of food protein for humans (Mamontova et al., 2019). Owing to the central role of metal-binding proteins in all biological processes, pulse proteins likely contain a suite of prospective element-binding peptides as storage organs. However, pulses are a common source of kinetically stable proteins and anti-nutritional compounds that precipitates elements during small intestinal digestion (Rousseau, Kyomugasho, Celus, Hendrickx, & Grauwet, 2019; Xia, Pittelli, Church, & Colón, 2016). In addition, the rigid cellular microstructure of pulses may also impede element dissolution from the source during digestion (Capuano & Pellegrini, 2019). However, these limitations may be overcome by food processing, as demonstrated by processing-induced enhancements in the bioavailability of endogenous elements in pulses (Gharibzahedi & Jafari, 2017). It is thus hypothesised that specific protein-derived fractions within suitable pulses, when isolated and/or processed to reduce anti-nutritional properties, may behave as potential soluble ligands vehiculating elements that enhance their solubility, hence bioaccessibility.

In line with the six global nutrition targets identified by the FAO for 2025, three are directly linked to the nutritional adequacy of essential elements: reductions in the prevalence of anaemia in women, stunting in children under 5, and low birth weight. In particular, iron (Fe), zinc (Zn), and calcium (Ca) are crucial in supporting these interlinked targets (Nguyen et al., 2020). However, Fe and Zn are generally of greater concern in the

context of bioaccessibility during gastrointestinal digestion, especially in plant-based diets (Platel & Srinivasan, 2016). Further understanding of the dynamic interactions between these two elements and pulse protein fractions would create new avenues to address the functional issues associated with their nutritional deficiency.

1.1. Research Aim and Questions

The proposed project aims to better understand whether protein fractions from one or more of three Australian grown pulses can be utilised to deliver fortified Fe and/or Zn for enhanced bioaccessibility during simulated human digestion.

This aim is achieved by addressing the following questions:

1. Which pulse-derived protein fractions have the most potential to solubilise fortified Fe and Zn in a manner that is bioaccessible in the small intestine?
2. Which bioprocessing methods are effective at producing protein hydrolysates that enhance the bioaccessibility of the fortified element (Fe or Zn)?
3. What are the properties of the selected protein derivatives that promoted element (Fe or Zn) bioaccessibility?
4. What are some effects produced by unabsorbed Fe on the enteric microbiome (colon), in the presence of the selected protein derivative?

This thesis attempts to answer these questions through a series of empirical experiments, using simulated *in vitro* gastrointestinal digestion and colonic fermentation models. Following a literature review of the topic in Chapter 2, a brief discussion of the research design and preliminary studies are described in Chapter 3. The rest of the thesis is structured as follows:

- **Chapter 4** begins the investigation with the selection of an Australian-grown pulse in possession of protein fractions with higher digestibility in the presence of fortified iron and zinc.
- The selected pulse fraction is then subjected to various bioprocessing methods in **Chapter 5**, to examine their ability to enhance Fe and Zn bioaccessibility.
- In **Chapter 6**, the composition of the fraction exhibiting the optimal enhancement properties following bioprocessing is characterised.
- The final research chapter (**Chapter 7**) delves into the effects of the protein-derived fraction on a human-derived gut microbiome, in comparison to a conventional mineral salt supplement.
- **Chapter 8** concludes with a synthesis of the research conducted for this thesis, including its major contributions towards the field, and a summary of future recommendations.

Chapter 2. Literature Review

This chapter contains one review article published in *Comprehensive Reviews in Food Science & Food Safety*, covering the state of knowledge on the biochemistry and bioavailability of prospective plant-derived carriers for Fe, Zn and Ca. The version included has been peer-reviewed without editorial copyediting, typesetting and proofreading. The full reference can be found in the preface of the thesis.

Opportunities for Plant-derived Enhancers for Iron, Zinc and Calcium Bioavailability: a review

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ABSTRACT:

Understanding of the mechanism of interactions between dietary elements, their salts and complexing/binding ligands is vital to manage both deficiency and toxicity associated with essential element bioavailability. Numerous mineral ligands are found in both animal and plant foods and are known to exert bioactivity *via* element chelation resulting in modulation of antioxidant capacity or micobiome metabolism amongst other physiological outcomes. However, little is explored in the context of dietary mineral ligands and element bioavailability enhancement, particularly with respect to ligands from plant-derived food sources. This review highlights a novel perspective to consider various plant macro/micro-nutrients as prospective bioavailability enhancing ligands of three essential elements (Fe, Zn and Ca). We also delineate the molecular mechanisms of the ligand-binding interactions underlying mineral bioaccessibility at the luminal level. We conclude that despite current understandings of some of the structure-activity relationships associated with strong mineral-ligand binding, the physiological links between ligands as element carriers and uptake at targeted sites throughout the gastrointestinal tract still requires more research. The binding behaviour of potential ligands in the human diet should be further elucidated and validated using pharmaco-kinetic approaches and gastrointestinal models.

2.1. Introduction

Over twenty inorganic elements are essential to human health as dietary nutrients, facilitating an array of biological functions within the human body (White & Broadley, 2005). These essential elements function as structural elements of biomolecules and tissues, and some also with physiological functions. Examples include Ca, P and Mg in bone health; Fe, Zn and Cu as metalloprotein and enzyme cofactors; Ca and P as intracellular signalling messengers; Na, K, Ca, Se in redox and acid-base balance and K, P and Na in osmoregulation (Replogle et al., 2011). Despite their widespread presence in biological systems, essential element deficiency remains a highly prevalent global concern that affects up to a third of the world's population (Jariwalla et al., 2010). The physiological and metabolic impairments associated with element deficiency affects socioeconomic development and productivity at the individual, societal and national level (Troesch et al., 2015).

The bioavailability of an element is defined as the total fraction in a given food or diet that is available to the body for physiological function after ingestion (Guo et al., 2014; La Frano et al., 2014). Bioavailability is central to the development of cogent element fortification and supplementation strategies, as the absorption of elements are associated to dosage, chemical form, delivery matrix, and pharmacokinetics. These factors are central to element bioavailability from both dietary and supplemental sources, particularly with divalent cations (Rafferty, Walters, & Heaney, 2007). Similarly, elemental content is a poor indicator of bioavailability in food matrices (Nosratpour & Jafari, 2019), as other bioavailability factors in foods are involved, such as absorption enhancers and inhibitors.

Oral administration through dietary or supplement routes is considered the most acceptable approach to reverse mineral deficiency, but is limited by bioavailability barriers. Additionally, approaches to address deficiency through supplementary salts can produce adverse sensory effects and dose-dependent bioavailability issues (Wang, Li, & Ao, 2012), and some may cause toxicity from overdose (Zand, Christides, & Loughrill, 2015).

In the past decade, food-derived components as bio-based delivery systems for enhancing element bioavailability have been gaining increasing consumer attraction as desirable substitutes to element supplements (Guo et al., 2014). Some isolated food polymer fractions comprise physicochemical properties, such as binding, bulking and fermentation ability, solubility and gel formation, which can facilitate elemental release and absorption (Baye et al., 2017; Belitz et al., 2009). Fractions that may enhance elemental bioavailability have been characterized in a diverse number of novel food sources, such as calcium-binding peptides from milk (Cross et al., 2007), soybean (Lv et al., 2013) and shrimp (Huang et al., 2011). However, bioactive supplements from plant-derived sources have not been extensively explored (Eckert, Bamdad, & Chen, 2014; Eckert et al., 2016). Plant metabolites such as peptides and polyphenols are known for their element chelation properties, although studies generally demonstrate source-dependent and inconsistent effects on element bioavailability.

The essential dietary elements Fe, Zn and Ca are well documented in a global deficiency (Shenkin, 2008; Suzuki, Landowski, & Hediger, 2008) and are often associated with bioavailability issues rather than a lack of quantity, especially from plant sources (Drago,

2017; Vavrusova & Skibsted, 2014). The bioavailability of elements from animal foods are fairly adequate, however the majority of populations worldwide relies on plant-based diets, particularly in developing countries with lower income (Solomons, 2000). Fe, Zn and Ca intake are critical for optimal wellbeing of all individuals, but particularly towards adequate development in vulnerable populations including pregnant women and children, to prevent intergenerational cycles of micronutrient malnutrition (Keeley, Little, & Zuehlke, 2019). Furthermore, the prevalence Fe, Zn and Ca deficiency as a public health predicament may be exacerbated under the context of global climate change, where field studies under projected conditions have demonstrated reduced soil uptake of these elements into commonly edible crop tissues (Dietterich et al., 2015; Loladze, 2014).

Nutritional bioavailability enhancement using recipient food-grade complexes as delivery agents has been a subject of research in recent years (Liu, Ma, Gao, & McClements, 2017; McClements et al., 2015). This current review builds upon existing knowledge aiming to further examine the role of plant-derived fractions in the bioavailability of Fe, Zn and Ca. First, we discuss the fundamental antagonistic causes leading to reduced bioavailability of Fe, Zn and Ca. We then focused on the chemistry of plant biomolecules that may be naturally present or derived and that may help promote the absorption of elements from foods. We propose that the identified plant-derived element binding ligands would greatly benefit from the use of biorelevant simulations throughout all stages of the digestive tract, which would allow characterization of their abilities as element bioavailability enhancers. Finally, a general outlook and some practical limitations toward their application in food systems are described.

2.2. Overview of element bioavailability

2.2.1. The biological roles of Iron, Zinc and Calcium

Iron (Fe) functions as a bound factor of many proteins, and as an active site element for over 200 metalloenzymes implicated in the primary biological processes of mammalian cells (Sheftel, Mason, & Ponka, 2012). As a component of haemoglobin, Fe plays a vital role in the binding, transport and activation of O_2 and CO_2 for cellular respiration within the human body (Rouault, 2003). In healthy adults, 1–2 mg of Fe are absorbed per day with a circulating pool of ~3–5 g (Sebastiani & Pantopoulos, 2011). Up to 90% of daily Fe requirements are reported to be met endogenously, primarily through the recycling of haemoglobin from senescent red blood cells. However, the absence of adequate stores engenders Fe deficiency and its associated anaemia (Clark, 2008). Fe-deficiency anaemia is classified amongst the most prevalent Fe related disorders worldwide, imperilling approximately one billion people in both developing and developed nations (Balarajan, Ramakrishnan, Özaltin, Shankar, & Subramanian, 2011; Knai, Sharan, & Baltussen, 2004). The condition can impair individuals' cognitive and physical development and productivity, which leads to morbidity and mortality in susceptible populations including women, children, and consumers of plant-based diets (Li, Jiang, & Huang, 2017; Zimmermann, Chaouki, & Hurrell, 2005).

In juxtaposition with deficiency, adverse conditions involving non-hereditary Fe overload are also reported from supplemental salts and parenteral administration (Hira et al., 2018; Yilmaz & Li, 2018b). Although Fe absorption is strictly regulated by host feedback of body stores and requirements for erythropoiesis (Nicolas et al., 2002), the lack of a

physiological route for Fe excretion can result in gradual build-up of a non-chemically bound and reactive pool of Fe. Due to the high redox potential of free Fe, electron transfer processes that occur catalyse a chain of Haber-Weiss/Fenton-like reactions. The associated radical intermediates formed are known to be cytotoxic and highly oxidative, and instigate various dysfunctions associated with oxidative stress within the body (Adjimani & Asare, 2015; Fraga, 2005). An effective yet safe therapeutic window has thus been central to the development of strategies targeting Fe deficiencies, with food-derived Fe-sources recognised as nontoxic alternatives that may reduce their ability to act as pro-oxidants in generating free radicals (Eckert et al., 2014; Prentice et al., 2016).

Zinc (Zn) is required for a myriad of structural, catalytic and regulatory roles (Bel-Serrat et al., 2014), and it has an ability to bind to ~10% of proteins within the human body (Andreini & Bertini, 2012). While Fe is confined intracellularly with specific physiological roles, Zn is ubiquitously present as a co-factor and intracellular signalling ion in the functioning of more than 300 enzymes and 1000 transcription factors (Cherasse & Urade, 2017; Roohani, Hurrell, Kelishadi, & Schulin, 2013). In addition, recent evidence from meta-analyses is suggesting the role of adequate Zn status in the prevention of chronic diseases, such as diabetes mellitus (Fernández-Cao et al., 2019). Although global risks of inadequate Zn intake have been decreasing over the past decade, it is still projected to affect 16-17.5% of the world's population (Kumssa et al., 2015; Wessells & Brown, 2012). To date, reliable biomarkers to assess Zn status remain undefined (Wieringa, Dijkhuizen, Fiorentino, Laillou, & Berger, 2015). Whilst most cases of Zn deficiency are mild with little clinical signs, it is often coupled with acute respiratory conditions, pregnancy

complications, diarrhoea, and restricted growth in children (White & Broadley, 2011; Wilson, Grieger, Bianco-Miotto, & Roberts, 2016). The prevalence of inadequate intake amongst infants and children with pneumonia, diarrhoea or malaria has been estimated to contribute to 800,000 excess deaths (Caulfield, de Onis, Blössner, & Black, 2004). However, high Zn intakes may interfere with copper absorption (Fraga, 2005). Homeostatic adjustments of dietary Zn absorption and endogenous excretions ensue according to body demands, with multiple excretion pathways (Krebs, 2000). Although a substantial amount of endogenous Zn is reabsorbed within the gastrointestinal tract, low intake or prolonged marginal intake can augment Zn elimination (Roohani et al., 2013). Half of all Zn losses occur in the lumen, and is further exacerbated in those suffering deficiency-related diarrhoea (Brown, Peerson, & Allen, 1998).

Calcium (Ca) is the most abundant inorganic element in the human body, 99% of which are in the form of calcium phosphate in bones (Guo et al., 2014). Apart from its role in the development and maintenance of bone structure and health, Ca also participates in muscle contraction, blood clotting, enzyme regulation and intracellular metabolism (Lagarda, Cilla, & Barbera, 2016). As a result of insufficient dietary intake and bioavailability, an estimated 3.5 billion people are at risk of Ca deficiency (Kumssa et al., 2015). Sufficient dietary Ca prevents the occurrence of rickets in infants and children (Sun et al., 2016), and is particularly critical between adolescence and 30 years old as one reaches peak bone mass (Hallberg, Rossander-Hulten, Brune, & Gleerup, 1992). This reduces the likelihood of osteoporosis during later adulthood, as skeletal demineralization gradually occurs (Drago, 2017).

Similar to Fe and Zn, Ca absorption and resorption are regulated by several host factors including the amounts ingested. Hormonal regulation through calcitriol [$1,25(OH)_2D_3$] in conjunction with parathyroid and thyroid hormones play a critical role in Ca homeostasis (Diaz de Barboza, Guizzardi, & Tolosa de Talamoni, 2015). Ca losses from both endogenous (salivary, biliary and pancreatic fluids) and dietary pools occur in the digestive lumen and *via* faecal excretion (Drago, 2017). However, cases of hypercalcemia and hypercalciuria have been common from excessive intake of supplements (Ross, Taylor, Yaktine, & Del Valle, 2011). The dysregulation associated with both deficient and excess quantities of Ca are linked to the pathogenesis of inflammatory, degenerative and metabolic diseases (Peterlik & Cross, 2009), as well as an increased risk of some cancers (Barry et al., 2017).

Both Fe and Ca are physiologically classified as type one nutrients, with inadequacies initially leading to reductions in body stores prior to affecting functional components; while Zn is a type two nutrient where declines in plasma levels are observed following observations in clinical symptoms (Kondaiah, Yaduvanshi, Sharp, & Pullakhandam, 2019). This reveals that clinical Zn deficiency can be particularly difficult to detect, due to the absence of an established biomarker (Brown et al., 2004). Nonetheless, considering the dire consequences in physiological imbalances of Fe, Zn and Ca, understanding the factors that control the bioavailability of these elements are crucial for addressing their deficiency and toxicity issues. The following sections will briefly introduce the chemistry of Fe, Zn

and Ca as found within food matrices, and how their speciation determines bioavailability through the digestive tract.

2.2.1. The chemistry of Iron, Zinc and Calcium from food sources

2.2.1.2. Iron

Iron is primarily found in two readily reversible oxidation states in foods: Ferric (Fe^{3+}) and ferrous (Fe^{2+}). As a transition metal, Fe shows oxo- and thiophilicity with its ability to form coordination complexes with O-, S- and N- atoms on donor ligands (Kepp, 2017). According to Cremonesi, Acebron, Raja, and Simpson (2002), Fe in solution exists as the di- or trivalent ion in an aqua complex under anoxic conditions. However, the ferrous-aquo complex is rapidly deprotonated at intestinal pH in the presence of oxygen, forming precipitates of ferric-oxo-hydroxides. Fe is highly prone to complexing with other compounds of plant origin that can inhibit its uptake, whilst Fe of animal origin is usually complemented with factors that circumvent Fe precipitation and associated bioavailability issues. These absorption promotors from animal sources are collectively termed ”meat factors”, and knowledge regarding their enhancing mechanisms are briefly explored in the current text in Section 3. Haem/heme, the form of organic Fe that accounts for 40% of the total Fe found in animal foods (Saunders, Craig, Baines, & Posen, 2013), is a principal component. Haem is a complex of ferrous Fe coordinated to a protoporphyrin ring and is highly bioavailable (Li et al., 2017), and constitutes more than 40% of total absorbed Fe in meat-eating populations (Egli & Hurrell, 2010). Some plants also have haem group proteins, such as the leghemoglobin found in some plant roots (Kannan, Sithara, &

Chandru, 2015). However, these root sources are not considered as a nutritional iron as the roots are not conventionally consumed.

Other naturally occurring bound forms of Fe include phytin in plants, and ferritin in both plant and animals. Fe-bound to phytin (myo-inositol hexakisphosphoric acid, IP), is considered to have little bioavailability in its native form (Sokrab, Mohamed Ahmed, & Babiker, 2014). This is due to its number of highly negatively charged phosphate groups on phytin, which bind with high affinity to cations at neutral physiological conditions of the small intestine (~ pH 7) (Gibson, Raboy, & King, 2018). Ferritin is a ubiquitous family of Fe-storage polypeptides found in virtually all living organisms. The globular protein complex encapsulates a ferric Fe core, and has shown potential in some human studies to be a readily bioavailable form of Fe (Theil et al., 2012; Theil, Davila-Hicks, & Lönnerdal, 2004). However, differences in subunit compositions amongst food sources of ferritin may confer diverse properties in relation to its digestibility, and thus bioavailability (Lv, Zhao, & Lönnerdal, 2015). In a study by Jin, Frohman, Thannhauser, Welch, and Glahn (2008), both reconstituted plant- and animal- type ferritin from horse spleen apoferrin have been found to release their Fe core during *in vitro* digestion, and the Fe was subsequently prone to interactions with absorption enhancers such as ascorbic acid, and inhibitors such as phytate. For leghemoglobin, an investigation using Caco-2 cell cultures demonstrated that iron from partially or fully purified leghemoglobin has lower bioavailability compared to bovine hemoglobin (Proulx & Reddy, 2006).

2.2.1.3. Zinc

Zinc is a trace element and is widespread in foods due to its pervading role in biological systems. It has remarkably high binding affinity for food proteins and nucleic acids from various plant and animal food sources (Drago, 2017). In plants, a proportion of Zn is also present in insoluble phytin bodies together with Fe (Raes, Knockaert, Struijs, & Van Camp, 2014). Non-protein bound Zn is poorly understood in respect to ligand exchange, apart for its affinity for various inorganic and organic ions due to the lack of stereochemical preferences (Krężel & Maret, 2016). The best dietary sources of Zn are derived from meat and seafood flesh, both of which provide necessary quantities with high bioavailability (Fraga, 2005; Hambidge & Krebs, 2007). Adequate intakes are provided easily with meat inclusion in one's diet, yet much of the world's population subsists on plant foods as sole sources of Zn (Gibson, Bailey, Gibbs, & Ferguson, 2010). Concentrations found in plant products are considered insufficient even in sources containing high concentrations, such as grains and legumes. These foods are known to contain Zn-binding low molecular weight compounds, which can affect its bioavailability. For example, high molar ratios of phytic acid (0.66 kDa) to Zn are well known to inhibit Zn absorption from plant-based diets (Wessells & Brown, 2012). On the other hand, carrier proteins such as casein phosphopeptides and milk lactoferrin can act as ligands to solubilize zinc (Ainscough, Brodie, & Plowman, 1980; Delshadian et al., 2018).

2.2.1.4. Calcium

The chemical properties of Ca from different food sources play a critical role in its bioavailability. The primary dietary Ca contributors are dairy products, which intrinsically

possess high concentrations. Calcium absorption enhancers found in milk, such as lactose, organic acids, casein and whey proteins (Buchowski, 2015), make dairy-based matrices an efficient promoter of Ca bioavailability. Plant-derived Ca is present in vegetative or reproductive tissues (e.g. the leaves and seeds) and constitute its second highest dietary contributor (Dayod, Tyerman, Leigh, & Gilliham, 2010). However, dietary Ca bioavailability from plants is limited by its ability to readily form stable interactions with biomolecules in plants, which can act as absorption inhibitors. In plants, a large fraction of Ca can be found cross-linked in cell wall structures, or bound to various organic/inorganic anions as insoluble carbonate or oxalate salts (White & Broadley, 2003). In these complex structures Ca provides structural integrity to plant cellular membranes (Hepler, 2005), however, the amount of Ca that undergoes solubilisation from this composite matrix during digestion in the human digestive tract is limited. Additionally, solubilised free Ca^{2+} tends to precipitate in its carbonate form under the alkaline pH and aerobic conditions of the small intestine (Goss, Prushko, & Bogner, 2010). Ca bioavailability is thus challenging, despite its abundant distribution and high concentrations in plant-based foods.

2.2.3. Bioavailability during transit through the gastrointestinal tract

2.2.3.1. Element absorption during gastrointestinal (GI) digestion

For an element to be bioavailable, it must first be bioaccessible for absorption. The accessible element must cross the apical mucosal membrane lining the GI tract *via* a transcellular or paracellular route, and into the portal circulation (Harvey, 2001; Krebs, 2000). From the basis of their physicochemical properties, elements can diverge significantly in their ability to be transported across the columnar epithelial cells lining the

small (enterocytes) and large (colonocytes) intestines. Some general key intrinsic properties that affect their absorption and hence bioavailability include solubility in gastrointestinal fluids, chemical speciation and physical characteristics of the food matrix (Apostoli, 2006).

A number of dynamic digestive processes occur sequentially prior to impending absorption, beginning from oral processing. The oral route of element delivery takes place through the ingestion of food. Within the oral cavity, mastication contributes physically for size reduction of the food, and is assisted by salivary wetting and enzymatic amylolysis of starch (where present) by salivary α-amylase. The secretion of salivary proteins such as mucins may be important for element bioaccessibility from food matrices, as they not only contribute to eventual elemental dissolution but may also interact with molecules that act as element enhancers or inhibitors (Çelebioğlu, Lee, & Chronakis, 2019; Delimont, Rosenkranz, Haub, & Lindshield, 2017). The bolus then travels down the oesophagus passing the oesophageal sphincter into the stomach. Here, an amalgamation of proteolytic enzymes, acidic secretions and physical churning takes place in a highly acidic environment of pH 1.7 – 2.8 (Dressman et al., 1990; Feldman & Barnett, 1991), which favours element dissolution and solubility (Miller, 2007). Its relevance to elemental bioavailability is, however, limited as little element uptake occurs during this stage in the stomach with exception of Cu^{2+} (Lowndes & Harris, 2005).

The bulk of element absorption occurs in the proximal section of the small intestine, the duodenum (Eckert et al., 2014), under neutral pH and aerobic conditions. The passage of

the bolus from the stomach lowers the fasting pH in the duodenum from pH 7.0 to 5.5, which transiently favours element solubility. However, the pH gradually rises to back to ~ 7.0 at the terminal ileum section due to bicarbonate secretion (Gharibzahedi & Jafari, 2017). These conditions can lower the amount of bioaccessible element through different ways. For example, a reduction in the concentrations of free ions arises in some elements, due to the formation of poorly soluble mineral oxides and hydroxides under oxygenated conditions. The neutral pH also facilitates the accumulation of element-organic complex of various dietary or endogenously secreted compounds that act as mineral ligands. Depending on their physicochemical properties, these ligands may reduce or enhance element solubility (Carbonaro, 2011).

2.2.3.2. Trans- and para-cellular element transport

Elements undergo an array of unique or shared transport pathways across the apical membrane of the enterocytes that form the small intestinal lining (Kiela & Ghishan, 2016). Transcellular and paracellular pathways are the two primary transport mechanisms, both of which are subject to regulation by nutritional and hormonal factors (Diaz de Barboza et al., 2015). The transcellular route is the predominant pathway where the intake is low, requiring active transport by specific membrane transporters on enterocytes surface and is thus saturable across a concentration gradient. However, different ions present in the lumen can be an imperative aspect in the active transport process. Some element transporters can be non-specific, where elements with similar coordination geometry and ionic radii can be competitive with each other with respect to transporter binding. This is exhibited by the interactions between Fe, Zn and Ca with the communal divalent metal transporter DMT-1,

that mediates the uptake of Fe^{2+} (Rouault, 2013) and Zn^{2+} (Espinoza et al., 2012). The affinity of DMT-1 towards various divalent cations (including Ca^{2+}) can demonstrate inhibition in the cellular uptake of these elements at certain concentrations (Shawki & Mackenzie, 2010; Thompson, Sharp, Elliott, & Fairweather-Tait, 2010; Yamaji et al., 2001). However, soluble mineral chelates can undergo transcellular absorption with separate transport pathways from those of the free ions. For example, an element-bound peptide can be absorbed intact by selective peptide transporters carrying the element with it (Chakrabarti, Guha, & Majumder, 2018). This route can provide circumvention of any competition between ions for metal-specific carrier transport.

The passive non-saturable paracellular route of Fe, Zn and Ca uptake typically occurs under high intake, where an influx of intraluminal saturation can overcome the lumen-positive transepithelial potential difference (Field, Harris, & Pollock, 2010). Passive uptake may also be mediated with the presence of certain osmotic agents, such as sugars (Buchowski, 2015; Christides & Sharp, 2013) and other soluble element binding ligands where elements can co-diffuse with water into the interstitial space (Goff, 2018). Membrane permeability between cell junctions can be modulated by the presence and expression of Claudin proteins at certain segments, which form channels that can facilitate selective ion uptake (Workinger, Doyle, & Bortz, 2018). Claudin expression by epithelial tissues is host-dependent (Tsukita, Tanaka, & Tamura, 2019), but is also mediated by the presence of gut bacteria metabolites such as short-chain fatty acids (SCFAs) derived from dietary fibre (Zheng et al., 2017).

2.2.3.3. Apical Iron, Zinc and Calcium influx

The route of Fe uptake is highly contingent on its chemical form and elemental speciation. Haem bound ferrous iron is readily absorbed as a haem-Fe^{2+} soluble complex conceivably involving a haem carrier protein (HCP-1). Ferric-containing ferritin is also able to enter the enterocyte intact, *via* an unspecified, saturable form of receptor mediated endocytosis (Kalgaonkar & Lönnerdal, 2009; San Martin et al., 2008). Free ferric Fe is only able to undergo transcellular absorption after its reduction to ferrous Fe, either by dietary components such as organic acids and phenolics, or through an intestinal ferric reductase (duodenal cytochrome b) at the brush border of duodenal enterocytes (Frazer, Wilkins, Vulpe, & Anderson, 2005). Reduction of the ferric ion by dietary reducing agents is conditional; for example, reduction by ascorbic acid only takes place below pH 6 (Hsieh & Hsieh, 1997). Transport into the cell takes place primarily through the action of the DMT-1 for intracellular utilization or storage (Fuqua, Vulpe, & Anderson, 2012). Due to the harmful nature of free Fe as an oxidative catalyst, absorption of Fe ions *via* passive diffusion is limited (Geisser, 2007). On the other hand, precipitated forms of ferric Fe salts are unavailable for uptake and are passed to the colon where dybiosis is experienced as a result of the iron-mediated growth of pathogenic bacteria.

Zn^{2+} ions are absorbed through binding to endogenously secreted ligands such as mucins (Food and Agriculture Organization & World Health Organization, 2004). The carrier-mediated route is considered to be the primary pathway, with paracellular uptake ensuing at higher intake (Krebs, 2000). Apical transport is thought to be mediated by the protein Zip4 (SLC39A4), which is upregulated in response to high luminal zinc concentrations (Dufner-

Beattie et al., 2003; Weaver, Dufner-Beattie, Kambe, & Andrews, 2007). Zn uptake is not generally considered to be different from organic versus inorganic sources within a food matrix, although more efficient uptake is observed from animal-based sources (King & Keen, 1999). Zinc-amino acid conjugates are known to be taken up by amino acid carriers and is mostly uninhibited by complexing agents (Sauer et al., 2017). While Fe homeostasis is structured by controlling absorption, Zn is regulated through the excretion of endogenous Zn (Kondaiah et al., 2019), as well as luminal and cytoplasmic zinc concentrations (Maares & Haase, 2020).

Small intestinal absorption of soluble Ca^{2+} is an intertwined process between transcellular and paracellular pathways. The paracellular route prevails with normal to high intake, which occurs from the duodenum to the ileum (Kiela & Ghishan, 2016). An estimated 65% of total Ca absorption takes place in the ileum, due to the relative length of the segment. Claudin proteins (Cldn-2, Cldn-12, and Cldn-15) are known to facilitate absorption through enhancing Ca^{2+} permeability in the jejunum and ileum (Fujita et al., 2008). The transcellular pathway of apical transport is upregulated at lower Ca intake, predominating in the duodenum and jejunum (Christakos, 2012). Active Ca transport is also dependent on calcitrol, which plays a role in the biosynthesis of the Calbindin-D9k that binds to Ca^{2+} ions and mediate cellular transport from the apical to the basolateral side (Bronner & Pansu, 1999). A number of other duodenal transporters have been identified to work synergistically towards apical Ca intake, including TRPV5, TRPV6, and Cav1.3 (Wongdee & Charoenphandhu, 2015). Transcellular transport is dependent on 1,25-dihydroxyvitamin

D3 [1,25(OH)2D3], which upregulates the expression of these calcium transporter genes (Balesaria, Sangha, & Walters, 2009).

2.2.3.4. Element uptake in the colon

Insoluble elements in their matrix-bound, organically complexed or precipitated forms, which escape absorption in the small intestine are passed into the colon along with other undigested food and indigestible matter. The colonocytes transport mostly water, electrolytes and prebiotic microbial fermentation products such as SCFAs (Fairweather-Tait & Johnson, 1999), and is generally considered to be a limited site for element uptake. The more constricted colonic epithelial cell junctions impose greater difficulties in expediting paracellular uptake, although non-direct means of element uptake by the colonocytes may be facilitated through anion exchange and separate transporters (Kiela & Ghishan, 2016). The factors controlling colonic elemental absorption are not well understood in humans, with limited studies demonstrating evidence of element uptake in nutritionally significant amounts, except for Ca, Mg and possibly Fe (Alexander, Swanson, Fahey, & Garleb, 2019). Large intestinal absorption of Ca^{2+} has been suggested to account for ~10% of total calcium uptake (Bronner & Pansu, 1999), occurring *via* both active transcellular and passive diffusion routes in the rectum and distal colon (Thompson, Wolever, & Trinidad, 1996). Ca-SCFA complexes have been proposed as a route to paracellular absorption through anion exchange mechanism (Hara, 2002). Colonic Fe availability is restricted (Parmanand et al., 2019; Swinkels, Kortman, Tjalsma, & Raffatellu, 2014), likely as a protective mechanism against free Fe-induced oxidative damage and inflammation (Yilmaz & Li, 2018a), and their utilization by pathogenic bacteria (Grinter, Walker, & Milner, 2013).

Evidence has been reported in the literature regarding Fe and Zn caecal absorption in rodents (Bouglé et al., 2002; Seal & Mathers, 1989). However, there isn't enough information regarding the extent to which this occurs in humans (Carvalho et al., 2017; Gopalsamy et al., 2015).

There is accumulating evidence from both human and rodent studies suggesting the stimulation of element absorption by prebiotic fermentation products, particularly SCFAs such as acetate, butyrate and propionate (Scholz-Ahrens, Schaafsma, van den Heuvel, & Schrezenmeir, 2001; Whisner & Castillo, 2018). SCFAs may mediate localised reductions in luminal pH (Topping & Clifton, 2001), supplementing the reduced (anaerobic) environment of the colon (Hedrich, Schlömann, & Johnson, 2011), and augment extracellular reductases' activity (Swinkels et al., 2014; Takeuchi et al., 2005) in enhancing ion solubility. In rodents, SCFAs have been linked to increases in Fe and Ca transporter expression (Carvalho et al., 2017; Fukushima, Aizaki, & Sakuma, 2008), as well as gas-induced distention of the mucosal surface area for absorption (Scholz-Ahrens et al., 2016).

2.3. Ligands in relation to bioavailability

Cations such as Fe^{2+}, Zn^{2+} and Ca^{2+} may form reversible ion-pairs or chelates with numerous endogenous molecules from the body or derived from foods. The food ligands are ordinarily characterized as compounds with a minimum of two element binding sites from electron donor atoms that coordinate to the central elemental ion. Under favourable conditions, proton displacement by the element ion can occur in the presence of acidic groups (e.g. -OH, -COOH, -SH, -NOH) and Lewis bases (e.g. -C=O, $-NH_2$, and other

electron pair donors) of various biomolecules found in food (Kontoghiorghe, Kolnagou, & Kontoghiorghes, 2015; Lindsay, 2017). Regardless of the chelating ligand, the strength of binding plays an important role in mediating elemental bioavailability. Loosely coordinated complexes may dissociate during digestion, allowing for precipitation or ligand exchange prior to prospective uptake. Strong complexation may lead to elimination in the case of an insoluble element-ligand complex, or reduced intracellular efflux following uptake with a soluble element-ligand complex. In addition to being soluble, an effective chelate needs to be able to bypass co-precipitation with other insoluble complexes.

Although the literature has largely emphasized the strong mineral-ligand binding in bioavailability enhancement, weak mineral-ligand pairs that dissociate under gastric digestion may still facilitate element bioavailability through re-complexation *in situ*. This is largely determined by the availability of free ligands, dissolution/reformation kinetics and gut transit time (Harju, 1989). The presence of free gastric-dissociated soluble ligands, particularly in excess molar ratios, may encourage immediate mineral-ligand complexation and directly undergo uptake. Where complexation occurs during early small intestinal digestion (i.e. duodenum and/or jejunum), uptake is propagated at peak efficiency and likely faster than insoluble ligands can bind to the element ion (Vavrusova, Raitio, Orlien, & Skibsted, 2013). The lag phase before precipitation, in which complex recombination can occur has been demonstrated by kinetic studies on Ca salts (Skibsted, 2016). Ca salts that rapidly reform soluble complexes in the duodenum, such as those with hydroxycarboxylic acids, often demonstrate higher fractional absorption and retention *in vivo* (Costello, Franklin, Reynolds, & Chellaiah, 2012; Hackl et al., 2016). Similarly, a

proposed mechanism of the 'meat factor' as a Fe bioavailability enhancer is the fast formation of soluble ferrous complexes with both gastric-hydrolysed and cysteine-containing peptide fractions during early protein digestion (Hurrell, Reddy, Juillerat, & Cook, 2006; Kapsokefalou & Miller, 1995; Mulvihill, Kirwan, Morrissey, & Flynn, 1998; Tabatabai, Swain, & Reddy, 2002). Similarly, studies by Huh, Hotchkiss, Brouillette, and Glahn (2004); Laparra, Tako, Glahn, and Miller (2008) reported that specific carbohydrates originating from glycosaminoglycans of muscle tissues can enhance Fe bioavailability. Transit time, a factor preponderating these interactions, is also commonly considered in the absorption of Fe (Leary et al., 2017), Zn and Ca (Fleet & Schoch, 2010), despite limited literature towards its role in bioavailability enhancement *via* foods. For Fe and Zn where efficient uptake is limited to within the small intestine, lack of ligand dissolution during its transit time may be unfavourable for elemental bioavailability. Such low bioavailability would be observed for soluble ligand complexes that provide progressively latent release, or for strongly bound insoluble ligand complexes that evade small intestinal dissolution. On the contrary, weak chelation sufficient to facilitate the dissolution of ions in solution can enhance element bioavailability (Lönnerdal, 2000).

The prevailing factors under physiological conditions that affect element-ligand complexes have been summarized in **Figure 1**. Purified biomolecular ligands have the potential to negate the bioaccessibility complexities of food matrices, while circumventing the toxicity of supplementary salts. Although there is broad literature consensus of many food compounds augmenting or inhibiting element bioavailability within the context of food matrices, the processes underlying the absorption, distribution, metabolism and excretion

(ADME) of isolated food fractions remain obscure. Element-ligand interactions are largely considered as Lewis acid-base interactions (Lundgren & Stradiotto, 2016). For a given ligand, the hard and soft character are thus important dictators in the properties of complex formation. Ca^{2+} and Fe^{3+} are hard acids with affinity for ligands of the same property, while Fe^{2+} and Zn^{2+} are borderline acids. However, Fe^{3+} as a transition element with higher charge density possesses different coordination properties to Ca^{2+}. Subsequently, whilst all three element present similarities in binding target preferences, the stability, thermodynamic and kinetic affinity of bound complexes can diverge significantly. Some prospective element-ligand interactions between food components for Fe, Zn and Ca are summarized in **Table 1**.

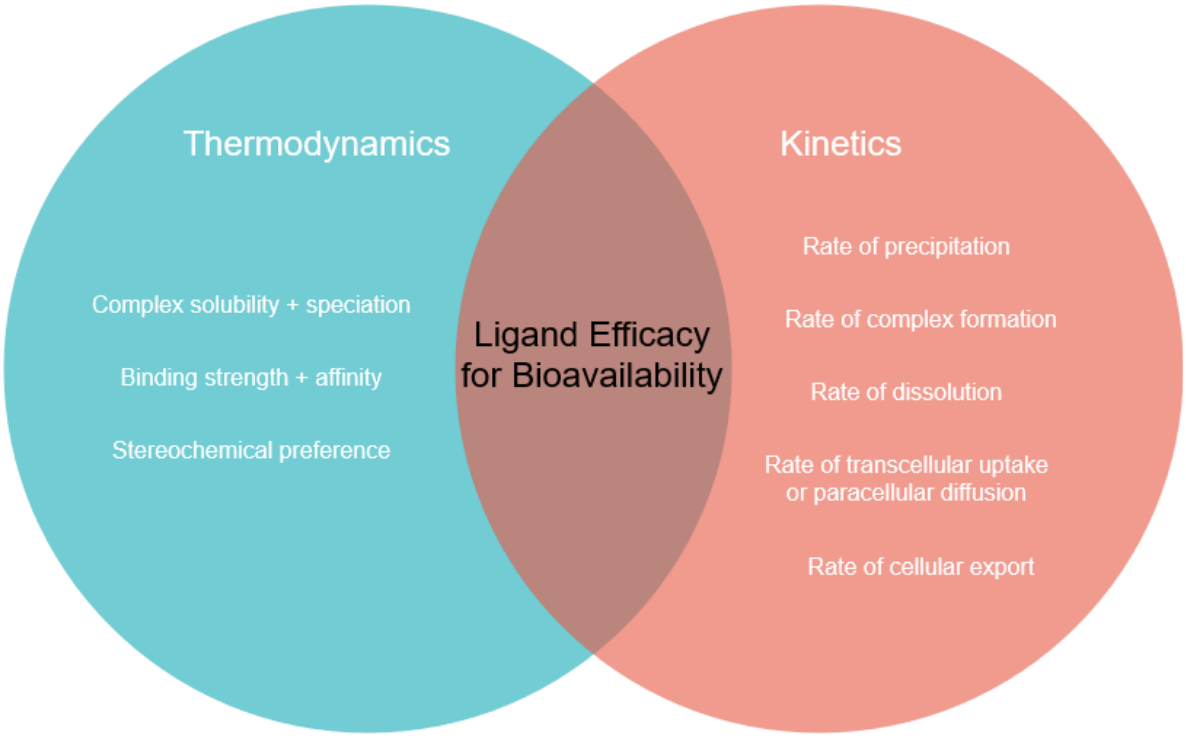


Figure 1. Determinants of ligand efficacy for mineral bioavailability

Table 1. Targeted elements and their known interactions with food-derived ligands, in correspondence with *in vivo* bioavailability responses

Element	Proteins	Carbohydrates	Other metabolites	Reported prospective enhancers *in vivo*	Reported putative inhibitors *in vivo*
Fe^{2+}/Fe^{3+}	Binds to N-terminus and sulphur residues of AAs/peptides, haem, phytoferritin, transferrin, nicotinamine, mucins	Binds to lignin and hemicellulose, lactose, ferric ion co-complexed with phytate	Binds to oxides/hydroxides, organic acids, phytic acid, tannins (catecholic and gallolic complexation), monosaccharides, polyols, carbonates, phosphates, acidic phospholipids, free fatty acids	Ascorbic acid, muscle tissue, glycosaminoglycans, vitamin A and carotenoids, non-digestible carbohydrates	Phytic acid, polyphenols, Calcium, proteins
Zn^{2+}	Binds to acidic side chains, phosphorylated residues, deprotonated nitrogen and sulphur residues of AAs/peptides, ferritin, serum albumin, nicotinamine, mucins, metallothioneins	Binds to charged polysaccharides (COOH groups), co-complexed with phytate	Binds to oxides/hydroxides, organic acids, phytic acid, phosphates, tannins (labile complexation)	Vitamin B6, animal proteins, cholesterol, dairy	Phytic acid, Calcium, Iron, Tin, proteins (bovine serum albumin, dephytinized soy), maillard products

Element	Proteins	Carbohydrates	Other metabolites	Reported prospective enhancers *in vivo*	Reported putative inhibitors *in vivo*
Ca^{2+}	Binds to acidic side chains, phosphoserine moieties of AAs/peptides, phytate-protein complexes, phytoferritin	Binds to acidic groups of pectins (and other charged polysaccharides), OH and COOH groups of lignin	Binds to oxides/hydroxides, organic acids, phosphates, phospholipids, free fatty acids, phytic acid	Lactose, prebiotic oligosaccharides, phosvitin peptides	Dietary fiber, phytate, oxalate

Table contents have been revised from (Watzke, 1998). Reported dietary modulators are derived from comprehensive reviews on Fe, Zn and Ca (Bel-Serrat et al., 2014; Meacham, Grayscott, Chen, & Bergman, 2008; Skibsted, 2016).

The following sections delineate the features of some macro- and micromolecules that have been identified to interact with elements, and likely leverage their bioavailability. Additionally, the direct and subsidiary means in which these compounds may modulate elemental absorption are highlighted.

2.3.1. Protein/Peptide derivatives

Proteins, peptides and amino acids (AAs) possess multiple ligand groups for element interactions, are amphoteric by their nature and able to react both as a base and as an acid. Considering that approximately 30% of biological proteins are metal- or metalloid-bound (Hauser-Davis & Parente, 2018), peptide conjugation is a broadly employed method for enhancing the cellular and nuclear entry of various biomolecules including elements (Dinca, Chien, & Chin, 2016; Puckett & Barton, 2010). The ability of protein fractions to confer elemental bioavailability stems from their element solubilizing or reducing properties at the small intestinal pH values, and/or as barriers shielding element ions from binding to other insoluble ligands. Element binding proteins and their derivatives from various food sources diverge in structure and composition.

2.3.1.1. Bioactive protein hydrolysates

Food-derived bioactive peptides are short amino acid sequences of 2 to 20 residues (Zarei et al., 2016), which can occur naturally or upon release from its native protein *via* heat or enzymatic processing. The element-binding property of bioactive peptides may accompany a spectrum of physiological activities observed with their ingestion (Bünning & Riordan, 1985; Carrasco-Castilla et al., 2012); including antioxidant, antimicrobial,

immunomodulatory and antihypertensive activities (Chakrabarti et al., 2018; Xia, Bamdad, Gänzle, & Chen, 2012). Certain AA motifs may also exert element bioavailability enhancement within the gastrointestinal tract (GIT). Depending on the AA composition and sequence, element chelation can be achieved *via* specific or non-specific binding sites of residues with electron and ionic-rich side chains (Cai, Klamczynska, & Baik, 2001; Walters, Esfandi, & Tsopmo, 2018), or encased sterically through conformational states (Kozłowski, Bal, Dyba, & Kowalik-Jankowska, 1999). The presence of the terminal carboxyl and amine groups in all AAs and peptides are electron pair donors for coordinate bonding. The amine ligand in particular only possess one lone paired electron, which forms complexes weaker than the oxygen ligand that has two (Kawaguchi, 1988). Nonetheless, these are thus considered poor element coordination ligands relative to some functional side chains of individual AAs such as aromatic rings, which can convey more stable coulombic and non-coulombic interactions with the coordinated element (Apostoli, 2006). A peptide's native AA sequence and orientation may adopt certain conformations that may also contribute to strong element binding by directional effect towards coordinating orbitals of the central element ion (Guo et al., 2014; Walters et al., 2018).

As supplements, synthetic mineral-amino acid chelates have been found to achieve higher bioavailability than inorganic salts. For example, high bioavailability of synthetic Fe-AA chelates was reported by Jiménez-Alvarado, Beristain, Medina-Torres, Román-Guerrero, and Vernon-Carter (2009), and also those of Zn-AA (Hennigar & McClung, 2018) and Ca-AA chelates (Ashmead, 1991). However, the high energy required for free AA transport can lead to issues with competitive uptake, saturation and osmosis (Clemente, 2000).

Additionally, some AA-based chelators (e.g. ferrous bisglycinate) have been found to be poor at inhibiting complex formation with other ligands (Mellican, Li, Mehansho, & Nielsen, 2003). These issues have prompted great interest in using protein hydrolysates as element carriers instead. Compared to chemical methods, enzymatically hydrolysed proteins have the advantage of higher structural integrity and controlled hydrolytic sites (Clemente, 2000; Walker & Sweeney, 2002). The end product can generate a mix of free AAs and peptides that utilise respective pathways within the GIT, which leads to more efficient transport with less saturation (Rerat, Nunes, Mendy, & Roger, 1988). Both peptides (di-, tri- and oligo-) and small polypeptides may cross the intestinal wall intact utilising transporters or non-carrier dependent mechanisms (van der Wielen, Moughan, & Mensink, 2017), with some transporters possessing superior efficiency to those of AA transporters (Li et al., 2017). Subsequently, given the requisite that the element-bound peptide fractions are themselves bioavailable, they can effectively deliver elements across the epithelial wall. Compared with native food proteins hydrolysates may also overcome protein allergenicity concerns and high resistance to proteolysis during transit in the digestive tract.

While co-ingestion of some soluble peptides has been demonstrated in human studies to enhance acute intestinal absorption of Fe (Taylor, Martínez-Torres, Romano, & Layrisse, 1986), Zn (Lönnerdal, 2000), and Ca (Hunt, Johnson, & Fariba Roughead, 2009; Kerstetter, O'Brien, Caseria, Wall, & Insogna, 2005), the efficacy of peptide-element chelates as supplements has only been examined *in vitro* and in animal models, with the exception of milk caseinophosphopeptides (CPPs) (Ait-Oukhatar et al., 2002; Miquel & Farré, 2007)

and lactoferrin (Dix & Wright, 2018). To date, progress towards food-derived peptides to enhance element bioavailability has been focused on the identification of Fe-, Zn- and Ca-chelating peptides and motifs from numerous food sources, most which are of animal origin (Walters et al., 2018; Wang et al., 2012). Whilst only some authors have examined the digestion of element-bound peptides *in vitro* or by rodent models, most studies have been pivotal in unravelling specific peptide properties or AA sequence associated with enhanced chelation activity. For comprehensive lists of food-derived, element binding hydrolysates, readers are referred to reviews for Zn (Udechukwu, Collins, & Udenigwe, 2016), and Fe/Ca (Sun et al., 2016; Walters et al., 2018). Some of the element binding properties identified in these food-based studies have been summarized in **Table 2**.

Table 2. Some peptide properties attributed to enhanced chelation activity or enhanced bioavailability of divalent cations, from various food sources

Properties		**Mineral**	**Additional notes**	**Source**
Functional groups	Thiol group (Cysteine, glutathione)	Fe/Zn	Chelating or reducing Ferric Fe Electron rich	(Agostinho, de Souza Oliveira, Anunciação, & Santos, 2016; Martínez-Torres, Romano, Layrisse, & Taylor, 1986; Pace & Weerapana, 2014; Sun et al., 2017; Tabatabai et al., 2002; Walters et al., 2018)
	Imizadole group (Histidine)	Fe/Zn	Electron rich	(Sun et al., 2017; Tabatabai et al., 2002; Zhang & Allen, 1995)
	Carboxyl group	Ca	Bound Ca ↑ with hydrolysate COOH content	(Bao, Lv, Yang, Ren, & Guo, 2008)
AA residues	Asparagine and Glutamine residues	Ca/Fe/Zn	Chelation *via* carboxylate or NH groups in the side chains	(Cortivo, Castellani, Martelli, Michelotto, & Abatangelo, 1982; Daengprok et al., 2003; Gerbino, Mobili, Tymczyszyn, Fausto, & Gómez-Zavaglia, 2011; Miquel, Alegría, Barberá, & Farré, 2005; Sun et al., 2017)

Properties		Mineral	Additional notes	Source
	Serine and phosphoserine residues	Ca		(Sun et al., 2016)
	Phosphorylated residues	Ca/Fe/Zn	Chelation sites for + charged ions	(Bouhallab & Bouglé, 2004; Miquel et al., 2005; Zhong et al., 2016)
	Arginine	Fe	Chelation *via* ↑ - charge	(Sun et al., 2017)
	Methionine	Fe/Zn	Chelation *via* hydrophobic residues or carrier-mediated processes	(House, Van Campen, & Welch, 1996, 1997)
Other properties	Molecular weight	Fe	MW > 10 kDa ↑binding	(Seth & Mahoney, 2000)
		Fe	MW < 10 kDa ↑binding	(Storcksdieck, Bonsmann, & Hurrell, 2007)
		Fe	MW < 5 kDa ↑binding	(Torres-Fuentes, Alaiz, & Vioque, 2012)
		Fe	MW < 1 kDa ↑binding	(Agostinho et al., 2016; Torres-Fuentes et al., 2012; Xia et al., 2012)
		Ca	MW 5-10 kDa ↑binding	(Eckert et al., 2014)
		Ca	MW >10 kDa ↑binding	(Lv, Bao, Yang, Ren, & Guo, 2008)
	Charge and hydrophobicity		↑ binding	(Liu, Bao, Lv, Xu, & Guo, 2012; Sun et al., 2017)

A compilation of studies on soluble cereal and legume protein hydrolysates in relation to their element binding and reducing properties are presented in **Table 3**. These studies demonstrated a trend of chelation capacity being generally enhanced by sub-fractionation, and a moderate degree of hydrolysis (~ 7-39%). These processes are often associated with an increase in the availability of electron-rich AA residues, which effectively facilitate binding interactions and complex stability. Moreover, synergistic effects between charged and hydrophobic/aromatic groups have also been observed by Eckert et al. (2014) and Pownall, Udenigwe, and Aluko (2010). It has been suggested that the presence of charged side chains initially attract element ions through electrostatic interactions and coordinate bonding, where its positioning within the peptide can be further stabilized by hydrophobic interactions and pi-stacking of aromatic rings (Eckert et al., 2014). Although a linear correlation between binding activity and the proportion of AAs with charged residues have been documented (Bao et al., 2008; Lv et al., 2013), both compositional and conformational differences are significant determinants of complexation due to possible steric effects. This explains the finding that the highest Fe-chelating activity in un-fractionated hydrolysates, despite identifying that fractionation enhanced concentrations of hydrophobic AAs (Pownall et al., 2010).

In congruence with element-chelating peptides from other food sources, a varying range of MW have been found to be effective for element chelation. Although optimal molecular weight (MW) values in relation to binding activity can be concluded from **Table 3** (e.g. 0.5-1.0 kDa for Fe binding, 8.0-14.4 for Ca binding), studies by Eckert et al. (2014) have found distinct solubility differences amongst ultrafiltered peptide size fractions complexed with

Fe, Zn and Ca, which were highly dependent on the enzyme chosen for protein hydrolysis. This means that for each element, the ideal MW range offering the prime balance between binding capacity and stability is likely unique and it also depends strongly on amino acid compostion and position.

Most studies, however, examined element chelation as a function of the *in vitro* antioxidant capacity exerted by the purified hydrolysate, which only provide information on the structural properties that promote element binding under the physicochemical parameters examined. The GIT, *in situ* presents significantly different environments as endogenous secretions containing salt and electrolytes affects element binding and binding strength (Foltz, van der Pijl, & Duchateau, 2009). As an example, the presence of chloride anions in gastric and intestinal fluids (Mudie, Amidon, & Amidon, 2010) have been found to increase the binding affinity of Ca in CPPs (Recio, Guerra, Torrado, & Skibsted, 2019). It would thus be useful to re-evaluate the identified binding properties using dynamic *in vitro* simulations or animal models, prior to harnessing these characteristics in the design and optimization of soluble element-complexing agents for bioavailability.

Table 3. Legume and grain-derived peptide fractions with their chelation structure-activity relationships

Source	Protein fraction/s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Chickpea (Cicer arietinum)	Purified peptide hydrolysate	Fe and Cu	Purification: Immobilized metal affinity chromatography (IMAC) Fractionation: Gel filtration (GF) chromatography	- Purified subfractions show ↑chelation activity vs. hydrolysates - ↑ Fe chelating activity: High His content (20-30%), small molecular size (< 500 Da)	Sequential pepsin and pancreatin (37 °C, pH 2.5 for 180 min and 7.5 for 180 min, respectively)	Megías et al., (2007); Torres-Fuentes et al., (2012)
Common bean (Phaseolus vulgaris)	Crude protein isolate-hydrolysate and phaseolin	Fe and Cu	Fractionation: Size exclusion chromatography (SEC)	- ↑ Fe chelation activity: 0.7–1.0 kDa fractions (protein isolates), and < 0.43 kDa (phaseolin)	Sequential pepsin and pancreatin (37 °C, pH 2.5 for 90 min and 7.5 for 120 min, respectively)	Carrasco-Castilla et al. (2012)

Source	Protein fraction/s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Cowpea (Vigna unguiculata)	Desalted protein concentrate-hydrolysate	Fe and Cu	Fractionation: *GF* chromatography	- ↑ chelating capacity from hydrolysates obtained with Alcalase, Flavourzyme and sequential Alcalase-Flavourzyme vs. Pepsin-Pancreatin	Respective systems: Alcalase (pH 8) and Flavourzyme (pH 7), at 50°C, 90 min Sequential systems: Alcalase-Flavourzyme (50°C, 45 min) and Pepsin-Pancreatin (37°C, pepsin – 45 min, pH 2, pancreatin – 45 min, pH 7.5, total: 90 min)	Segura-Campos, Ruiz-Ruiz, Chel-Guerrero, & Betancur-Ancona, (2013)
Adzuki (Vigna angularis)	Protein fractions (albumins, globulins, prolamins and glutelins), desalted via dialysis (MW cut-off 5 kDa, < 7 kDa)	Fe and Cu		- Highest peptide levels/soluble peptides were obtained from albumin - Concentration of soluble peptides↓in the order of albumin > glutelin > crude > globulin > prolamin - Globulin fraction showed highest Fe chelation activity	Sequential system: oral digestion (15 min total, 37°C), gastric (2 h, 37°C), intestinal (1 h, 37°C)	Durak, Baraniak, Jakubczyk, & Świeca, (2013)

Source	Protein fraction/s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Pea (Pisum Sativum)	Protein isolate-hydrolysate, ultrafiltered (MW < 3 kDa)	Fe	Separation: RP-HPLC	- Hydrolysis ↑ concentration of BCAAS and Phen - Fractionation ↑ concentration of hydrophobic AAs (BCAAS, Trp, Phe) - ↑Reducing power in fraction with non-polar, aliphatic AAs (Pro, Val, Ile, Leu, Phe) - Fe-chelating activity highest in un-fractionated hydrolysate (95%) - Metal chelating activities showed strong positive correlations with total aromatic AAs and total % of hydrophobic AAs	Thermolysin (3 h, 55°C, pH 8.0)	Pownall et al., (2010)

Source	Protein fraction/s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
African Yam Bean (Sphenostylis stenocarpa)	Protein isolate, protein isolate-hydrolysate and ultrafiltered fractions (< 1 kDa – 10 kDa)	Fe		- Peptides < 1 kDa ↑ ferric reducing power, attributed to total hydrophobic/aromatic AAs - No significant difference between Fe-chelating activity of fractions	Alcalase (pH 8, 4 h at 50°C)	Ajibola, Fashakin, Fagbemi, & Aluko, (2011)
Broad Bean (Vicia Faba)	Protein isolate-hydrolysate	Fe		- Alcalase produced the best effect for Fe-chelation (88.22%) - Degree of hydrolysis (DH) was correlated with chelation activity	Respective systems: Papain (pH 9, 60°C), neutral protease (pH 7, 50°C) and Alcalase (pH 8, 50°C) for 4 h	Li, Ding, Han, & Chen, (2015)
Winged bean (Psophocarpus tetragonolobus)	Protein isolate-hydrolysate	Fe	Separation: RP HPLC	- Fe-chelating activity was correlated with DH - Fraction containing acidic and basic residues ↑ Fe chelating activity	Papain (pH 6.5, 70°C) for 14 h	Yea et al., (2014)
Mung bean (Vigna radiata)	Protein isolate-hydrolysate	Ca, Fe		- DH not directly linked to Ca and Fe-binding ability - ↓ Ca and Fe-binding as DH increased above	Respective systems: Alcalase, Flavourzyme (both at pH 8, 50°C), trypsin, pancreatin	Budseekoad et al., (2018)

Source	Protein fraction/s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
				certain values (DH = ~39%)	(both at pH 7, 37°C), pepsin (pH 2, 37 °C), mixture of pancreatin and Alcalase (50:50), mixture of pancreatin and Flavourzyme (50:50), (both at pH 8, 50 °C). All were 8 h	
Soybean (Glycine max)	Protein isolate-hydrolysate (3 –10 kDa)	Ca	Purification: IMAC	- Bound Ca was related with Glu, Gln, Lys and Pro content	Sequential: Protease M (pH 3, 50°C for 60 min), glutaminase (pH 7, 50°C for 180 min)	Lv et al., (2013)
Soybean (Glycine max)	Isoflavone-rich isolate, protein isolate-hydrolysate	Ca		- Max. binding capacity between 10-30 min of hydrolysis - % yield of Ca-peptide complex maximum at 120-180 min - Protease M hydrolysate has a high level of Ca affinity	Respective systems: Protease M (pH 4.5 and 50°C), Alcalase (pH 8 and 60°C) for 0 - 240 min	Wang, Lu, Yang, Wang, & Yang (2017)

Source	Protein fraction/s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Soybean (Glycine max)	Protein isolate-hydrolysate	Ca	Fractionation: SEC	- Ca content in hydrolysates not significantly affected by phytate - Protease M produced maximum levels of Ca binding - Peptides with ↑ Ca binding capacity had average MW of either 14.4 kDa or 8 to 9 kDa - Ca binding capacity linearly correlated with -COOH content	Respective systems: Protease M (pH 3, 50°C), pepsin (pH 2, 37°C), Neutrase and Flavourzyme (pH 7, 50°C), all for 60 min	Bao et al., (2008)
Soybean (Glycine max)	Protein isolate-hydrolysate	Fe	Separation (phytate removal): HPAE	- Dephytinisation ↓ degrees of hydrolysis - Moderate DH = ↑ chelation activity (6.79%)	Respective systems: trypsin, pepsin (37°C, pH 8 and 2, respectively), Protemax, Flavorzyme, Neutrase and Alcalase (50°C, pH 7.5, 7.1, 7.1 and 8, respectively), all for 120 min	Zhang, Huang, and Jiang (2014)

Source	Protein fraction/s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Wheat (Triticum aestivum) germ	Germ protein isolate-hydrolysate	Zn	Separation (desalting): IMAC	- Max. chelating activity found between 150-200 min hydrolysis - MW 1221 Da was best chelate of Zn - Simulated gastrointestinal digestion of peptide–zinc complex markedly higher than inorganic Zn salt (Caco-2 cell uptake)	Respective systems: Alcalase (pH 8.3, 55°C), Flavourzyme (pH 7, 60°C), papain (pH 7, 55°C), up to 400 min Sequential systems: pepsin-pancreatin gastric (2 h, 37°C), intestinal (2 h, 37°C),	Zhu, Wang, and Guo (2015)
Wheat (Triticum aestivum)	Germ protein isolate-hydrolysate	Ca	Separation: ultrafiltration, HPAE, fractionation: GF chromatography, RP-HPLC	- Binding capacity with DH ↑ markedly until 240 min, then ↓ rapidly - Asp and Thr possess major binding sites	Alcalase (pH 8, 50°C, 50-300 min)	Liu, Wang, Wang, and Chen (2013)

Source	Protein fraction/s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Barley (Hordeum vulgare)	Hordein hydrolysate	Fe, Zn, Ca	Separation: IMAC	- A mixture of varying low MW peptides, and a combination of charged/hydrophobic residues are synergistic in ↑ binding	Respective systems: Alcalase, (pH 8, 50°C), Flavourzyme (pH 7, 50°C), pepsin (pH 2, 37°C), trypsin (pH 7, 37°C), up to 2 h	Eckert et al. (2014)
Barley (Hordeum vulgare)	Glutelin hydrolysate	Fe	Separation: RP-HPLC	- DH and hydrophobicity not directly linked to Fe chelation - Highest Fe chelation found in fraction < 1 kDa (50 – 67% hydrophobic AA residues) - Fraction with M_w > 10 kDa showed good reducing power comparable to ascorbic acid	Respective systems: Alcalase (pH 8, 50 °C), Flavourzyme (pH 7, 50°C), up to 4 h	Eckert et al. (2016)

Source	Protein fraction/s assayed	Element/s assayed	Peptide fractionation method	Characteristics contributing to high chelating activity	Enzymatic hydrolysis conditions	Source
Rice (Oryza sativa)	Protein isolate-hydrolysate	Fe		- Pancreatin digestion ↑ Fe chelating activity to a greater extent than pepsin digestion - Hydrolysates from pepsin–pancreatin digestion exhibited a time-dependent ↑ in reducing power	Sequential system: gastric (2 h, 37°C), intestinal (4 h, 37°C),	Liu, Wang, Li, Liang, and Yang (2016)
Rice bran (Oryza sativa)	Defatted rice bran isolate-hydrolysate	Fe		- Optimal chelation of peptides from combined enzymatic systems for moderate time (90 min) - Hydrolysates ↑ solubility/retention/transport/uptake compared to unhydrolysed protein (Caco-2) - Extended hydrolysis inhibited these parameters	Respective and combined systems: Alcalase (pH 8, 50°C), Flavourzyme (pH 7, 50°C), up to 4 h Sequential system: Alcalase (pH 8, 50°C) 1 h, Flavourzyme (pH 7, 50°C) 3 h All subjected to simulated digestion: gastric (1 h, 37°C), intestinal (3 h, 37°C)	Foong, Imam, and Ismail (2015)

2.3.1.2. Phytoferritin

Ferritins are a family of Fe-storage proteins found in the storage organelles of various organisms. All ferritins possess 24 subunits arranged in a hollow shell, with a cavity of 80 Å that allows the formation of an inorganic mineral complex (Harrison & Arosio, 1996). By nature, the ferritin complex binds up to 4500 ferric atoms in its interior as $Fe(OH)_3$ salt, although chemically prepared ferritin containing Zn and Ca in the core have also been synthesized (Li, Viravaidya, & Mann, 2007; Li, Zhang, Yang, Zhao, & Xu, 2014) and Zn (Zhen et al., 2013). Both *in vitro* and *in vivo* studies have demonstrated that the intact ferritin polypeptide, when bioaccessible, undergoes endocytosis by intestinal cells (San Martin et al., 2008; Theil et al., 2012; Theil et al., 2004). However, elemental bioavailability may be compromised if the element is released due to structural instability of the protein.

The elemental carrying capacity and structural stability of ferritins vary amongst sources. Animal ferritin is highly stable and has a slow gastrointestinal release of Fe (Mertz & Theil, 1983), thus effectively shielding the Fe against chelating ligands such as phytic acid and tannins in the GIT (Kalgaonkar & Lönnerdal, 2008). With plant-based phytoferritin, there have been mixed reports on its stability against gastric proteases, storage and heat-induced denaturation (Hoppler, Schönbächler, Meile, Hurrell, & Walczyk, 2008; Liu & Theil, 2005). Phytoferritin architecturally resembles animal ferritin, although animal ferritin consists of heavy (H-) and light (L) MW subunits, the latter of which is absent in phytoferritin (Zhao, 2010; Zielińska-Dawidziak, 2015). Phytoferritin is known to have one to three H- subunits (Zhou, 2017), which shares approximately 40% sequence identity with animal ferritin

(Zhao, 2010), but is subject to pronounced inter-species variation in its ratio and AA sequence (Roschzttardtz et al., 2013). These H- subunits are precursors to a specific domain extension peptide (EP) on the protein exterior that stabilizes its oligomeric conformation (Li et al., 2009; Yang, Zhou, Sun, Gao, & Xu, 2015). Conformational changes in the EP domain would easily catalyse structural dissociation, which can occur during food processing and gastrointestinal digestion (Hoppler et al., 2008), or during ambient storage due to the EP's serine protease-like activity (Yang et al., 2010). The digestive stability of the phytoferritin complex thus depends on the ratios between the different subunits (Liao, Yun, & Zhao, 2014).

Studies conducted on purified legume seed phytoferritins and their mineral bioavailability or stability are summarised in **Table 4.** Previously, multiple studies have been conducted on whole food matrices under the assumption that most of the seed iron existed as phytoferritin. However, contemporary quantification methods revealed that ferritin comprise approximately 18-69% of seed Fe, subject to high taxonomic dependence (Cvitanich et al., 2010; Hoppler et al., 2014; Hoppler, Zeder, & Walczyk, 2009). The forms of the remaining Fe vary depending on the source, and its cellular localisation and form can be modulated by physiological activity or food processing (e.g. soaking (Cvitanich et al., 2010)). Mineral bioavailability data from whole food studies have thus been excluded from the table, as well as former studies that have utilized radioactive labelling. For example, the earlier extrinsic radioactive techniques were not included in table 4, since they were recognized to result in incomplete isotopic exchange between the isotopic label and Fe

within the food (Consaul & Lee, 1983; Glahn, Cheng, & Giri, 2015; Jin, Cheng, Rutzke, Welch, & Glahn, 2008; Messina & Messina, 2010; Yang et al., 2015; Zhao, 2010).

Human *in vivo* studies mostly demonstrated that the absorption of purified soybean ferritin Fe was comparable to $FeSO_4$, an iron salt known for its efficient bioavailability (Zariwala, Somavarapu, Farnaud, & Renshaw, 2013). Phytoferritin in these studies were presumably intact, as Theil et al. (2012) showed through a simultaneous rodent study that phytoferritin endocytosis undergoes an independent, non-competing process from that of non-heme Fe. However, multiple *in vitro* studies have also shown that purified phytoferritin undergoes gastric dissociation and the released Fe interacts with various ligands (Bejjani, Pullakhandam, Punjal, & Nair, 2007; Hoppler et al., 2008). Plausible scenarios such as better digestive efficiency of *in vitro* environments and re-folding of dissociated oligomeric phytoferritin within the small intestine have been proposed (Zielińska-Dawidziak, 2015). Alternatively, since the $Fe(OH)_3$ core of phytoferritin is insoluble at intestinal pH and thus unlikely to be absorbed, efficient uptake as ferrous Fe would still ensue if oligomeric phytoferritin-catalysed reduction occurs after ferric Fe release from the mineral core. The presence of phytoferritin hydrolysis products may also limit the complexation of Fe with other insoluble ligands.

Table 4. Studies conducted on mineral bioavailability or stability of purified legume seed phytoferritins

Source	Form	Method	Element /s assayed	Findings	Interacting ligands tested	Source
Lupin (Lupinus augustifolius)	Purified	*In vivo*	Fe	- Statistically significant ↑ in serum parameters of healthy/anaemic rats	N/A	Zielińska-Dawidziak et al., (2012)
Soybean (Glycine max)	Purified	*In vivo*	Fe	- Phytoferritin and $FeSO_4$ are equally bioavailable in healthy/anaemic humans and rats	N/A	Lönnerdal, Bryant, Liu, & Theil, (2006); Theil et al., (2012); Theil et al., (2004)
	Purified and reassembled	*In vitro* Caco-2 cell uptake	Fe	- Ferritin degraded to varying extent under gastric digestion - > 50% of ferritin was intact after pepsin treatment for 45 min - Digestive stability depends on the ratio of rH-1 and rH-2 subunits - Tannic acid ↓ Fe bioavailability	Phytic acid, tannic acid, ascorbic acid	Jin, Frohman, et al., (2008); Lv et al., (2015)
	Recombinant H-2	*In vitro* digestion, *in vitro* release/ chelating activity	Fe	- Polyphenols (Gallic acid, methyl gallate and propyl gallate) with three OH groups can bind to rH-2 and ↑ digestion stability - Cinnamic acid derivatives ↑ Fe release in a structure-dependent manner		Sha, Chen, Zhang, & Zhao, (2018); Wang, Zhou, Qi, & Zhao, (2014); Wang, Zhou, Ning, & Zhao, (2016)

Source	Form	Method	Element /s assayed	Findings	Interacting ligands tested	Source
				- ↑ polyphenol chelating activity = faster rate of the Fe release		
Recombinant bean (Phaseolus vulgaris)	Purified	*In vitro*	Fe	- Ferritin-bound iron no longer detectable after boiling for 50 min - EDTA ↑ Ferritin-bound Fe after cooking	EDTA	Hoppler et al., (2008)
Pea (Pisum sativum)	Purified	*In vitro Caco-2 cell uptake*	Fe	- Fe core is released into digestive medium - Pea ferritin uptake inferior or indifferent to $FeSO_4$ - Ascorbic acid ↑, while phytic acid ↓ ferritin uptake	Phytic acid, ascorbic acid	Bejjani et al., (2007); Perfecto et al., (2018)
	Purified	*In vitro* autodegradation	Fe	- Thermal treatment at 60 to 80 °C can ↑ storage stability to 10 days minimum - Thermal treatment > 90 °C changes ferritin structure		
Adzuki (Vigna angularis)	Purified	*In vitro* autodegradation	Fe	- Adzuki phytoferritin exhibits higher stability than soybean rH-1 - The position Ser 68 is important in the control of protein stability	N/A	Li, Yun, Yang, & Zhao (2013)

Source	Form	Method	Element /s assayed	Findings	Interacting ligands tested	Source
Broad bean (Vicia faba)	Purified	*In vitro* autodegradation	Fe	- Stability of broad bean phytoferritin > soybean and pea ferritins	N/A	Yun et al., (2012)
Chickpea (Cicer arietinum)	Purified	*In vitro* autodegradation	Fe	- CSF not degraded at 4 °C during 39 days (Stability > SSF, PSF, and BBSF under the same conditions)	N/A	Lv, Liu, & Zhao, (2014)
Lentil (Lens culinaris), chickpea (Cicer arietinum), mung bean (Vigna radiata), pea (Pisum sativum)	Purified	*In vitro* digestion	Fe	- Most Fe released occurred during 120 min gastric digestion - Red lentil had the best stability	N/A	Gesinde, Udechukwu, & Aluko, (2018)
Soybean apoferritin	Reassembled	*In vitro* Caco-2 cell uptake	Ca	- Nanocage encapsulated Ca undergoes separate uptake pathway (TfR1 receptor) compared to free Ca ions (DMT-1 related)	N/A	Li et al., (2014)

Investigations in recent years have found the stability of phytoferritin to be influenced by its interactions with other compounds, particularly with polyphenols. Polyphenol derivatives with consecutive polyhydroxy groups, from monomers such as gallic acid and (−)-epigallocatechin-3-gallate (EGCG), to oligomeric proanthocyanidins have been reported to enhance the gastrointestinal stability of phytoferritin *in vitro* (Deng et al., 2011; Wang et al., 2014; Wang et al., 2016). Polyphenols appear to mediate a protein-association effect on phytoferritin that is dose-dependent and irreversible. However, this is structure-dependent and contingent on the degree of protein-polyphenol interaction. Where there is greater affinity for polyphenol binding by minerals (i.e. under certain molar ratios and exposure of mineral *via* ferritin degradation), rapid mineral liberation can be initiated from the ferritin core, as found by some authors studying tannic acid and cinnamic acid derivatives (Li, Jia, Yang, Deng, & Zhao, 2012; Sha et al., 2018). Mineral liberation may have contributed to findings *in vivo* using an anaemic rat model, where proanthocyanidins have been found to inhibit Fe uptake from soy phytoferritin (Yun, Zhang, Li, Chen, & Zhao, 2011), revisiting the bioavailability discrepancies between *in vitro* and *in vivo* studies.

Knowledge governing various phytoferritins' structure-binding activity relationships can offer novel ways to enhance its stability as a mineral bioavailability enhancer. For example, several studies have demonstrated that in phytoferritins with two H- subunits (H-1 and H-2), the homopolymer of H-2 is less susceptible to degradation (Dong et al., 2008; Fu et al., 2010). Other features contributing to degradation and stability (e.g. H-subunit ratio (Lv et al., 2015), specific AA positioning within EP sequence (Li et al., 2013)) have also

been identified. These key determinants may be targeted in the synthesis and reassembly of mineral-containing phytoferritin. Additionally, there is vast potential in the exploitation of the inter- and intra-species variation in phytoferritin structure, in which novel characterisation from various unexplored plant sources may be valuable. Currently, most studies have only explored phytoferritin auto-degradation under storage. While processing approaches such as thermal treatment has demonstrated potential in enhancing storage stability (Tang, Yu, Chen, & Zhao, 2019), it is imperative to examine the stability of different phytoferritins under gastrointestinal conditions to understand their ADME properties as mineral carriers.

2.3.2. Plant phytochemicals

Plant materials are rich in primary and secondary metabolites that constitute the plant phytochemicals, and which possess functional groups that can interact with elements through coordination and other non-covalent interactions (Ejima, Richardson, & Caruso, 2017). Whilst plant phytochemicals are generally considered to be detrimental to mineral bioavailability (Rousseau et al., 2019), there have been some mixed findings between different phytochemicals, particularly where effects from vegetal matrices are eliminated. The following sections will discuss the several phytochemicals (polyphenols, beta-carotene and nicotianamine) in relation to their element chelation properties, and the existing evidence on their potential to enhance mineral bioavailability as individual fractions.

2.3.2.1. Polyphenols

Polyphenols are a heterogeneous group of phytochemicals widely distributed in plants that include phenolic acids and flavonoids (Tsao, 2010). Polyphenols are generally reputed to be inhibitors of mineral bioavailability due to their propensity to form insoluble element complexes or co-precipitates under neutral to alkaline conditions during digestion (Feitosa et al., 2018; Petry, 2014). Short-term isotopic *in vivo* studies demonstrate the inhibitory properties of polyphenol co-ingestion on non-haem Fe absorption (Gillooly et al., 1983; Kaltwasser et al., 1998; Petry, Egli, Zeder, Walczyk, & Hurrell, 2010; Samman et al., 2001). However, the mechanisms for the reduction in Fe absorption is not clear. For example, some polyphenols are known to inhibit the activity of digestive enzymes (Bennick, 2002; Manach, Williamson, Morand, Scalbert, & Rémésy, 2005), and thus it is possible that Fe remained complexed within the non-digested starch and protein molecules, rather than being complexed by the polyphenols. Moreover, insoluble complex formation involves ligand displacement from the coordinated element by polyphenols following gastric dissociation (Yang et al., 2014). Such ligand displacement by polyphenols may be conditional upon the presence and relative stoichiometry of certain other competing intestinal ligands generated from the partially digested food and/or microstructure (Bornhorst & Singh, 2014), and endogenous secretions released upon food ingestion. Indeed, these factors support evidence of food co-ingestion possibly hampering the oral bioavailability of certain polyphenols (Naumovski, Blades, & Roach, 2015; Takehiko, Hiromu, & Kazuhiro, 2012), and in some cases, the mineral itself, e.g. Zn (Bel-Serrat et al., 2014), and Fe (Moretti et al., 2006). Conflicting results on Zn and Fe bioavailability are found from rodent and long-term *in vivo* studies (Delimont, Haub, & Lindshield, 2017;

Delimont, Rosenkranz, et al., 2017) as well as Caco-2 cells *in vitro* (Kim, Ham, Bradke, Ma, & Han, 2011; Kim, Pai, & Han, 2011; Ma, Kim, Lindsay, & Han, 2011). Similarly in Ca, polymeric polyphenols such as tannins have been previously reported to affect Ca absorption in rodents (Chang, Bailey, & Collins, 1994; Mitjavila, Lacombe, Carrera, & Derache, 1977), although the phenolic compound genistein has been found to have anabolic effects on rodent femoral tissues *in vitro* (Yamaguchi & Jie, 2001). Human studies investigating the effects of polyphenols on Ca have been recently reviewed, with no detrimental effects on bone metabolism (Austermann, Baecker, Stehle, & Heer, 2019).

In recent years, some studies have recognized the disparity amongst heterogeneous classes of polyphenols in forming mineral complexes of varying solubility, as well as their prospective reducing properties on ferric Fe. Several authors have examined the subsequent implications on mineral bioavailability, using *in vitro* Caco-2 cell models. For example, Vlachodimitropoulou, Naftalin, and Sharp (2010) showed that the flavonoid quercetin can enhance extracellular Fe(III) reduction by ferric reductases and enhancing the import of Fe(II). Works by Hart and others (2017; 2015) and Wiesinger, Cichy, Hooper, Hart, and Glahn (2020) have characterized significant differences amongst different purified black and common bean polyphenols, where certain compounds (e.g. catechin and epicatechin) demonstrated promotive behavior in Fe cell uptake and ferritin formation regardless of Fe/polyphenol molar ratio. Some polyphenols are conditionally promotive at certain molar ratios (e.g. luteolin-7-glucoside, cyanidin-3-glucoside), while others are inhibitory to Fe uptake (e.g. malvidin), highlighting the importance of hydroxylation patterns of the ring structure in element binding. Hart, Tako, Wiesinger, and Glahn (2020)

further found a general association between Fe uptake and the ratio of promotor to inhibitory polyphenols in yellow bean seed coats, where inhibitory condensed tannins was able to proportionately counteract the promotive effect of kaempferol compounds. These binding patterns may also be dependent on the composition of the food matrix, as Laparra, Glahn, and Miller (2009) have observed an inhibition of Fe uptake by Caco-2 cells from tea extract (a source of catechin), but an increase with the catechin standard solution. Investigations by Lesjak and others (2019; 2014) have validated the increase in Fe cell uptake by isolated quercetin and its analogues, using the Caco-2 model concomitantly with rodents *in vivo*. Though the authors have also identified decreased basolateral cell efflux and expression of Fe-transporters, this effect was identified as structurally related to the analogue used. It is possible that the inhibition of Fe efflux is due to strong binding of quercetin to Fe under intracellular conditions, although this does not deduce the possibility of Fe undergoing cell efflux past the 3 h time period examined in the study.

There have been investigations of a similar type in the literature on Zn (Kim, Pai, et al., 2011). Fractionated polyphenols from various beverages have been shown to have neutral or enhancement effects on uptake by Caco-2 cells, with tannic acid and quercitin stimulating the uptake of zinc and no effect from others (Sreenivasulu, Raghu, & Nair, 2010). The study also found that polyphenols promoted the expression of metallothionein (MT), a regulatory Zn transporter associated with homeostasis. On the other hand, Kim et al. (2011) found a reduction in Zn apical uptake and transepithelial transport by purified grape seed extract, but not from green tea extract and EGCG. Dissimilar to the previous study, the authors found no significant changes in MT expression.

The findings above are generally supported by binding studies under non-physiological contexts, which demonstrate that the affinity of specific binding sites towards Fe and Zn ions are dependent on structural features, element ion properties and pH (Andjelković et al., 2006; Dimitrić Marković, Marković, Brdarić, Pavelkić, & Jadranin, 2011; Sroka & Cisowski, 2003; Wei & Guo, 2014; Yang et al., 2014). On the other hand, the chelation properties of Ca have been less explored due to weaker affinity of the phenoxide group towards alkaline earth metals (Hider, Liu, & Khodr, 2001), although binding with phenolic acids has been reported (Granieri et al., 2017). Structures such as the catechol moiety and the chroman C-ring -OH and -keto groups in flavonoids can provide bidentate coordination sites for elements (Andjelković et al., 2006). Subsequently, the structural differences amongst polyphenols (e.g. degree of hydroxylation, polymerization, glycosylation) can be important determinants of mineral binding capacity, stability constants and variable affinity for different ions. The stability of some mineral-polyphenol complexes has also been reported to differ under physiological pH (Kontoghiorghe et al., 2015).

Given the diverse chemical properties of polyphenols, further studies are required to elucidate if certain polyphenols can produce Fe and Zn bioavailability enhancement in the absence of food matrices, using biorelevant simulations. This is particularly important given that polyphenol bioavailability can differ between states of matter (i.e. solid and liquid forms) (Aguirre & Borneo, 2019; Conte et al., 2016), and the structural changes (e.g. de-conjugation of the glycosylated moiety) that occurs during passage thorough disparate segments of the GIT (Tsao, 2010). Polyphenols have also been identified to cross-link

intestinal mucins, which modulates barrier properties and thus nutrient absorption (Georgiades, Pudney, Rogers, Thornton, & Waigh, 2014). This may have bioavailability implications, as mucin-binding plays an imperative role in facilitating Fe, Zn and Ca uptake.

2.3.2.2. Carotenoids

Carotenoids from plants, such as β-carotene are dietary retinoids that undergo enzymatic transformation into vitamin A (Adjimani & Asare, 2015; O'Byrne & Blaner, 2013). Carotenoids possess extended conjugated double bonds, and/or aromatic rings or pyrones that allow π-electron delocalization, contributing as binding sites for Fe, Zn and/or Ca, or as catalysts for Fe reduction (Llansola-Portoles, Pascal, & Robert, 2017). Their aromatic rings may play an essential role in Fe solubilisation, where the carbonyl and hydroxyl groups confer additional polarity that contribute to increased affinity for lipid-water interfaces, despite the generally lipophilic nature of carotenoids (Horiuchi et al., 2015).

Various *in vitro* studies have reported some evidence of direct metal-carotenoid or vitamin A interactions at physiological pH 6-7, with Fe, Zn and Ca. A list of some carotenoids in relation to binding and/or bioavailability of divalent cations are provided in Supplementary Data 1 (**Table 20**). García-Casal et al. (2000) found a significant enhancement in Fe uptake from Caco-2 cells with the presence of β-carotene, which effectively prevented the formation of insoluble complexes with phytate or polyphenols. A similar enhancement in Fe cell uptake with β-Carotene was found in an inflamed version of the model by Katz et al. (2015), where effectual intracellular release was additionally observed. Other authors

have found an increase in *in vitro* Fe and Zn bioaccessibility from dialysis following simulated gastrointestinal digestion (Gautam, Platel, & Srinivasan, 2010), or Fe in the Caco-2 model (Gargari, Razavieh, Mahboob, Niknafs, & Kooshavar, 2006) and dialysis (Singh, Bains, & Kaur, 2016). On the other hand, a series of investigations by Corte-Real et al. (2017; 2015) concluded that Ca precipitated carotenoids under simulated gastrointestinal digestion. The same authors found also that Zn binding to carotenoids was negligible, unless very high concentrations were present.

Similarly, human *in vivo* studies, have generally presented favorable effects of carotenoids on Fe/Zn bioavailability (García-Casal et al., 1998; García-Casal, 2006; Jimenez et al., 2010), however, other studies have found no influence from carotenoids (Chen et al., 2014; Walczyk, Davidsson, Rossander-Hulthen, Hallberg, & Hurrell, 2003). As previously highlighted, food matrices may be one of the confounding influences contributing to mixed effects on mineral bioavailability *in vivo*. Additionally, receptor-mediated effects have been found from rodents, where enhancements in Fe bioavailability and retention were identified to be related to increased expression of genes involved in absorption (DMT-1, Dyct B, Ferroportin 1, ferritin) rather than direct metal-carotenoid interactions (Citelli, Bittencourt, Da Silva, Pierucci, & Pedrosa, 2012). These amalgamated effects are complications in identifying whether carotenoids can be enhancers of bioavailability in Fe and Zn, and the degree in which possible promotive effects are related to its chelation properties. Additional investigations, particularly complementary to *in vivo* and *in vitro* studies are required prior to identifying carotenoids as possible Fe and Zn promoting agents. Similarly, the behavior of carotenoid-Ca complexation also requires further substantiation.

In vitro binding studies under simulated gastrointestinal conditions may be valuable in confirming structure-activity properties of carotenoids with more relevance to digestion conditions. For instance, the partial delocalization of the pyrone oxygen in xanthophylls may be of significance as a element binding site, as it is consistently in its non-fused form in carotenoids. This is in congruence with the *in vitro* experiments by Garcia et al (1998; 2006), who discovered increased Fe solubility with cyclic xanthophylls (β-carotene, lutein and zeaxanthin) compared to non-cyclic lycopene. In the same study, the authors corroborated this relationship using Fe isotope labelling in humans *in vivo*, albeit in the presence of food matrices. Another observation that is possibly structure-related is the ability of carotenoids to consistently surpass those of Vitamin A in its bioavailability-enhancing effects, in both *in vivo* (García-Casal et al., 1998; García-Casal, 2006; Jimenez et al., 2010) and *in vitro* (García-Casal et al., 2000; Katz et al., 2015) studies. While various carotenoids and Vitamin A bear high resemblance in chemical structure, the longer hydrocarbon chain lengths in carotenoids that provide increased contiguous element coordination sites may explain this phenomenon.

2.3.2.3. Nicotianamine

Some soluble, low MW element-binding ligands involved in plant mineral metabolism and transport have received some attention in bioavailability enhancement. An example is nicotianamine (NA), a non-specific element chelator known to accumulate in high concentrations in legume seeds including chickpea (Tan et al., 2018) and soybean (Nozoye et al., 2014). NA is known for its high affinity for divalent element cations, particularly

ferrous iron (Fe^{2+}) whilst its biosynthetic precursor, 2′-deoxymugineic acid (DMA), can chelate Fe^{3+} (Reichman & Parker, 2002; Tsednee, Huang, Chen, & Yeh, 2016). *In vitro* investigations utilizing simulated gastrointestinal digestion and the Caco-2 cell model have found both NA and DMA to be effective enhancers of Fe from biofortified wheat (Beasley, Bonneau, et al., 2019; Eagling, Wawer, Shewry, Zhao, & Fairweather-Tait, 2014). In particular, NA has been found to enhance Fe uptake to a greater extent compared to ascorbic acid, a known enhancer of Fe absorption enhancer (Beasley, Hart, Tako, Glahn, & Johnson, 2019). Several animal studies, including rodent (Lee et al., 2012) and broiler (Beasley et al., 2020) models have also found grains biofortified with NA to be highly bioavailable and effective at enhancing Fe status. Further studies are required to validate its potency as an enhancer of Fe bioavailability, which may possibly translate to other essential elements.

2.4. Outlook and potential limitations in food applications

Most of the plant-derived element binding molecules construed above are in their preliminary stages of characterisation as bioavailability enhancers. Some of the biomolecules have been investigated as stand-alone nutraceuticals in food applications, such as for bioactive peptides and polyphenols (Gonçalves, Martins, Duarte, Vicente, & Pinheiro, 2018). However, it remains undetermined to what extent the physicochemical or pharmacokinetic properties are modulated from being element-bound, and whether they would possess advantageous physico-chemical properties (e.g. better solubility) over currently employed supplementary mineral salts as directly consumed, or through excipient foods. Given current understanding of the absorption patterns at various sites for each

element in the human GIT, the element-bound compounds may benefit from being loaded through delivery systems that provide additional stability and loading capacity, protection from other competing insoluble ligands and controlled release. An example of this would be nano-encapsulation, a form of which has been demonstrated to enhance the bioavailability of ferrous glycinate in a rodent model, effectively circumventing phytic acid as a competing ligand (Yang, Yi, Li, & Ding, 2017). These strategies could be explored in the next stage of development suceeding confirmation of biological efficacy.

In this section, we highlight some prospective limitations in the application of these biomolecules towards addressing essential element malnutrition in the context of different global populations. These practical constraints must be carefully considered when applying these biomolecules as stand-alone or adjunct therapies to current strategies employed to increase essential element intake in humans.

2.4.1. Administration form and dosage

As with any essential element supplement or fortificant, a primary barrier towards biological efficacy inside the human body is influenced by the form of administration. Most micronutrient supplements are typically vehiculated in solid, liquid or powdered forms, and/or in food matrices (Oh, Keats, & Bhutta, 2020). This depicts that even if the soluble element chelators demonstrate effective utilization of Fe, Zn or Ca *in vivo*, the true biological effects (i.e. bioequivalence) may differ when administered alone as a supplementary compound or within different foods, due to disparities in bioaccessibility and bioconversion processes (Yetley, 2007). Even if the target compound exhibit excellent

bioavailability as supplements, incorporation into foods can be difficult due to changes in organoleptic properties, bioavailability and stability (Prentice et al., 2016). This is of particular concern with regards to at-risk populations in underdeveloped countries, where current micronutrient interventions typically involve biofortification of staple crops and home fortification. Element biofortification entails enhancements in the concentrations of both essential elements and the corresponding solubilizing biomolecule, typically in cereals and legumes, through genetic engineering, traditional plant breeding, and/or agronomic approaches (White & Broadley, 2005). Meanwhile, home fortification presents an approach where micronutrients (usually as a powder) are added to solid or semi-solid foods (De-Regil, Suchdev, Vist, Walleser, & Peña-Rosas, 2013; Dewey, Yang, & Boy, 2009). Through both approaches, the prevalence of monodiverse diets in low-income populations suggest that the element carrier may be highly prone to interactions with vegetal and fibrous food matrices, unless diet diversification or other absorption enhancers such as ascorbic acid are concomitantly applied. For example, although some flavonoids (e.g. epicatechin gallate) can form membrane-permeable complexes with iron that undergo transcellular uptake (Kim, Ham, et al., 2011), the presence of multiple *ortho*-substituted hydroxyl groups allows Fe to be co-complexed with poorly digested proteins and/or fibres in the plant matrix, forming aggregates that may be poorly soluble as discussed in section 3.2.1. Human studies have suggested the relative importance of variety selection in crop biofortification processes (Petry, Egli, Campion, Nielsen, & Hurrell, 2013; Petry et al., 2016), which takes into account of the various components of the plant matrix that also contribute to element bioavailability.

Moreover, food processing approaches may further modulate these interactions. For example, the maize germ fraction and the cotyledon cell wall of legumes have been identified as an physical inhibitor of Fe bioavailability (Glahn, Tako, & Gore, 2019; Glahn, Tako, Cichy, & Wiesinger, 2016). These studies indicate that processing techniques, which have the potential to disrupt the cell wall structure and fibrous fractions, may be an additional factor compounding the ability of soluble ligands to act as absorption promotors (Wiesinger et al., 2020). A successful example that resolves both factors can be found in the Manteca yellow bean (*Phaseolus vulgaris*), a phenotype with fast-cooking properties and promotive polyphenols (kaempferol flavonoids) that has been identified to have higher bioavailability through *in vitro* Caco-2 cell models and broiler studies (Wiesinger, Cichy, Tako, & Glahn, 2018; Wiesinger et al., 2019). Food processing approaches such as enzymatic hydrolysis and thermal, shear or pressure processing may also enhance the release of soluble ligands that may act as enhancers or negators from existing plant food matrices. For example, enzymatic hydrolysates can release peptides, whilst heating methods can facilitate the release of bound polyphenols and carotenoids within the cellular matrix (Gunathilake, Ranaweera, & Rupasinghe, 2018). Indeed, in circumstances where these biomolecules are vehiculated within the plant food matrix, food processing techniques may achieve a simultaneous reduction in some endogenously occurring anti-nutritional compounds occurring within the food matrix. A prominent factor impeding elemental bioavailability from plant-based foods is phytic acid, and its elimination may be attained by soaking, thermal processing, and enzymatic hydrolysis (Gupta, Gangoliya, & Singh, 2015). Subsequently, both *in vitro* and *in vivo* studies have demonstrated enhanced Fe and Zn bioavailability with phytic acid reduction (Liang, Han, Nout, & Hamer, 2008; Petry et al.,

2014; Petry et al., 2010; Vashishth, Ram, & Beniwal, 2017), which may be related to both reduction of inhibitors and release of enhancer molecules.

In relation to applications through home fortification, it is unknown as to what degree these biomolecules participate in some established interactions related to dosage and co-ingested compounds observed through existing supplemental approaches of Fe, Zn and Ca. The vulnerable populations targeted for interventions aimed at enhancing essential element intake often tend to experience severe multi-element micronutrient deficiencies, where multi-micronutrient supplementation strategies are suggested (Oh et al., 2020). This can modulate the potential of biomolecules to act as enhancers, exerting either positive or negative effects. In maternal women for example, the World Health Organization recommends both Fe and vitamin A (Tunçalp et al., 2017), which when co-administered, may interact to enhance Fe bioavailability as highlighted in Section 3.2.2. On the other hand, co-administration of multiple elements can lead to undesirable interactions during intestinal absorption. For instance, reviews of randomized controlled trials, animal and *in vitro* studies have identified reduced effectiveness of joint Fe and Zn supplementation, although the relationship can also be mutualistic depending on other factors such as therapeutic dose, element ratios, timing, and the form of vehiculation (Fischer-Walker, Kordas, Stoltzfus, & Black, 2005; Kondaiah et al., 2019). Similarly, Ca in the form of both food component and supplementary salts have been found to interefere with both haem and non-haem Fe absorption (Hallberg et al., 1992; Hallberg, Rossander-Hulthèn, Brune, & Gleerup, 1993) and status (Khan et al., 2014; van de Vijver et al., 1999) in humans, while no effects have also been reported (Abrams, Griffin, Davila, & Liang, 2001; Minihane & Fairweather-Tait, 1998). While the exact mechanisms may differ, the factors that affect

competition between Zn and Fe may also alter the direction and magnitude of interactions between Ca and Fe. This highlights a conditional nature in the efficacy of element bioavailability enhancers when co-administered with multiple micronutrients. Under such conditions the potential interactions at different doses, timing, and compatibility in common food matrices should be thoroughly examined through both *in vitro* and *in vivo* studies.

2.4.2. Legislative barriers

Biomolecules derived from plant foods offer great potential as a safe and biodegradable delivery system for stabilizing and enhancing essential elements' bioavailability. However, a prospective hinderence towards their application in more developed countries is the regulatory requirements associated with commercialization. Depending on their classification, there could be ambiguous boundaries as to how these biomolecules tailor into legislative frameworks at a country-level, and subsequently the parties responsible for managing their production (if permitted), distribution and quality control. These products may be classified as nutraceuticals, which are nutritional components that bring physiological or therapeutic benefits outside fundamental nutritional needs, and/or offer protection from chronic disease (Nasri, Baradaran, Shirzad, & Rafieian-Kopaei, 2014). In this case, the regulatory system may be under therapeutic goods amongst products such as complementary medicine and drugs, such as the Therapeutic Goods Administration (TGA) in Australia. In order to be lawfully supplied to consumers, the biomolecule would require registeration on an extensively regulated Register of Therapeutic Goods, and be produced in compliance with Good Manufacturing Practice (GMP) under the *Therapeutic Goods Act*

1989 (McEwan, 2007). On the other hand, if the biomolecule is to be provided and ingested through food matrices, such as through formulation of functional food products or biofortification through genetic modification, regulation of food standard and safety would be required. In Australia, this would be by Food Standards Australia & New Zealand (FSANZ). FSANZ operates within the Food Standards Code under multiple levels of governance, which comprises an independent set of legislations (*Legislation Act 2003*) related to matters prior, during and following food production (Ghosh, 2014). Separate agencies and departments at local and state government scales are often responsible for enforcement.

As such, these legislative frameworks can diverge greatly by nation. In the USA for example, the requirements applied to the novel substances that are added to either food or dietary supplements are monitored under the same system. Both applications are required to be subjected to review and approval by the Food & Drug Administration (FDA) as Generally Recognized as Safe (GRAS) prior to use (Food & Drug Administration, 2014). The FDA appointed a scientific committee that conducts independent risk assessments for novel ingredients or substances, which are available to the public through a GRAS Substances (SCOGS) Database. Similarly in the European Union, food-derived supplements or fortificants are considered as foodstuffs, and are regulated under the same system by the European Food Safety Authority (EFSA). Under the Directive 2002/46/EC, the EFSA permits the addition of Fe, Ca and Zn for nutritional purposes (Annex I), and provides an additional list of substances that are authorised as their carriers in Annex II (European Parliament, 2011). In China, novel nutrient supplements and fortifiers, including

mineral supplements are classified as health (functional) foods by the Chinese Food and Drug Administration (CFDA). The National Food Safety Standard of Health Food (GB 16740-2014) stipulates that the active ingredient and its excipient must comply with food safety standards and relevant provisions (Hu, 2017). However, all general, novel food compounds and new additives must first be assessed for safety and approved by the National Health and Family Planning Commission (NHFPC), prior to use as supplement or fortification compounds.

2.5. Conclusion

The identification, modulation and development of food-derived biomolecules as mineral bioavailability enhancers remain an unmapped and challenging field of study. This review has delineated the factors influencing luminal interactions between food ligands and three minerals (Fe, Zn and Ca). Additionally, it discussed some recent progress of understanding of mineral binding with nutritional fractions found in plant-based foods, many of which are found in legume seeds. In order to detect food-derived biomolecules that contribute to element bioavailability, it is important to understand the pharmacokinetic processes underlying currently effective soluble ligands for each element (e.g. citrate for Ca). Food matrix interactions and food processing strategies that may create and modulate the ability of certain ligands to act as bioaccessibility promoters, such as polyphenols or peptides, should also be thoroughly investigated. Whilst binding and release properties are important in the efficacy of element chelators, understanding these properties under biorelevant conditions of digestions from oral to intestinal segments will assist in the identification, development and validation of both novel and currently existing mineral bioavailability

enhancers. Soluble ligands utilized for element transport within plants, such as nicotianamine, have great potential to be explored in biofortification, food fortification or supplement strategies. However, their limitations regarding potential matrix interactions and legislative constraints must be overcome in order for these biomolecules to act as effective delivery agents for Fe, Zn, Ca, and possibly other essential elements. The use of plant-derived biomolecules as essential element bioavailability enhancers offer a novel avenue towards a generation of supplementary compounds, with potentially lower toxicity and greater ease of administration.

Chapter 3. Research Design and Approach

This chapter describes the conceptualisation of the experimental design behind the subsequent research chapters (Fig 2.), as well as the preliminary experiments involved in selecting the analytical methods. Namely, the extraction of pulse fractions to be examined in the initial simulated digestion study, and the selection and validation of some analytical methods utilised in the latter studies.

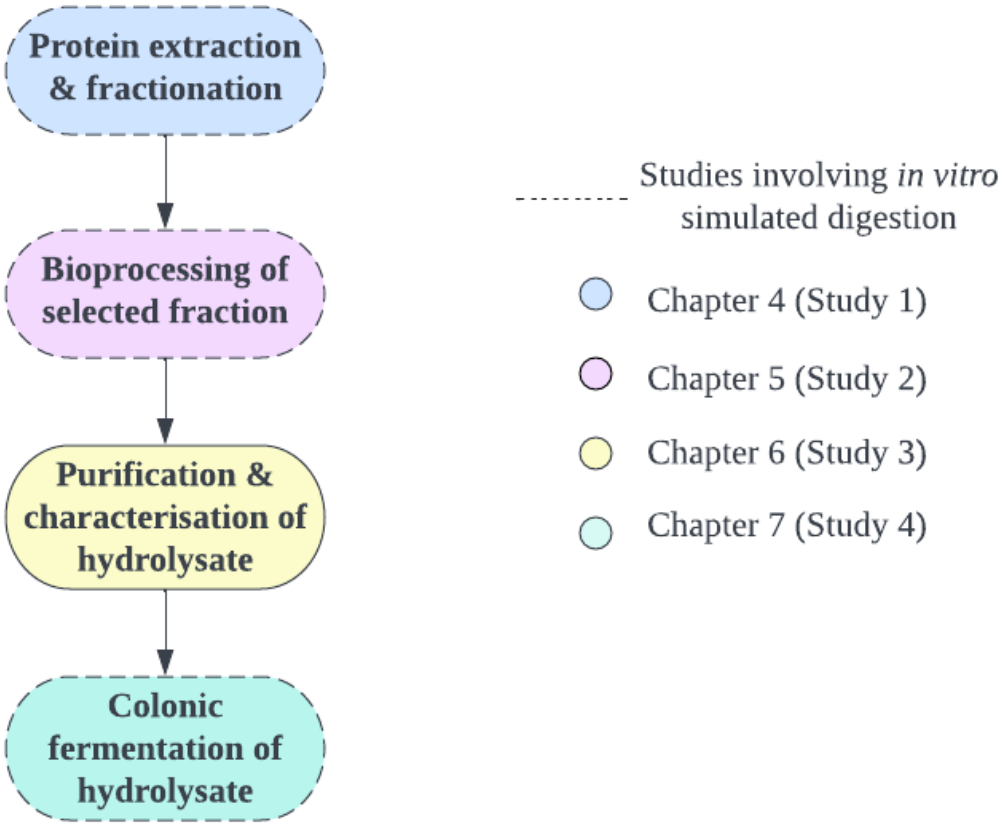


Figure 2. A flowchart of the structured study design of this thesis

3.1. Method development for protein fractionation, analysis, and processing

As described in Chapter 1, the current study centres the production of pulse protein fractions that may solubilise Fe and Zn mineral salts during the process of digestion. Pulses contain 17 – 40% protein by dry weight, the majority of which are seed storage proteins that are typically classified by their solubility (González-Pérez & Arellano, 2009). These include the albumins (water-soluble), globulins (salt-soluble), glutelins (alkali/acid-soluble) and prolamins (ethanol-soluble), which are extracted *via* the dispersion of ground particles in each of the solvents (Osborne & Campbell, 1898). This method allows for simple separation of the water- and salt-soluble fractions, which are of interest in this study as they are likely to be soluble under the pH-neutral and ionic conditions of the small intestine. However, the original fractionation method based on pure differential solubility is known to yield relatively impure fractions (Casey & Domoney, 1999). It is thus expected that without chemical additives (e.g. borates) in the solvent, the crude extracts will have some cross-contamination between these classes of proteins, as well as impurities such as non-starch polysaccharides (Rubio et al., 2014). Modifications in relation to solvent/substrate ratios, solvent concentrations, and desalting can be used to enhance extractability and reduce impurities (Gueguen & Cerletti, 1994).

This preliminary study aims to 1) determine a reliable soluble protein method suitable to quantifying the target analytes for the subsequent simulated digestion studies, and 2) optimise the processing conditions for the target protein fractions. The protocols used for these studies can be found in section 3.1.5. of this chapter (‘Protocols for preliminary experiments’).

3.1.1. Soluble protein analyses

As summarised in **Table 5**, three well-established colorimetric methods were examined. The selected assays are compatible with the NaCl used to extract globulins, and the target analytes (pulse proteins) do not possess key interference agents. The Biuret and Bicinchoninic acid (BCA) assays capitalise on the principle of cupric chelation by peptide bonds under alkaline conditions, whereas the Bradford assay uses the Coomassie blue dye that binds to proteins at acidic pH (Copeland, 1994). The original Biuret method is a robust assay, but has a limited working range of 5 to 160 mg protein per mL of sample. Meanwhile, the BCA assay provides higher sensitivity from absorption of the secondary complex between reduced cupric irons and the BCA reagent, leading to a lower but more dynamic range similar to the Bradford assay (**Table 5**).

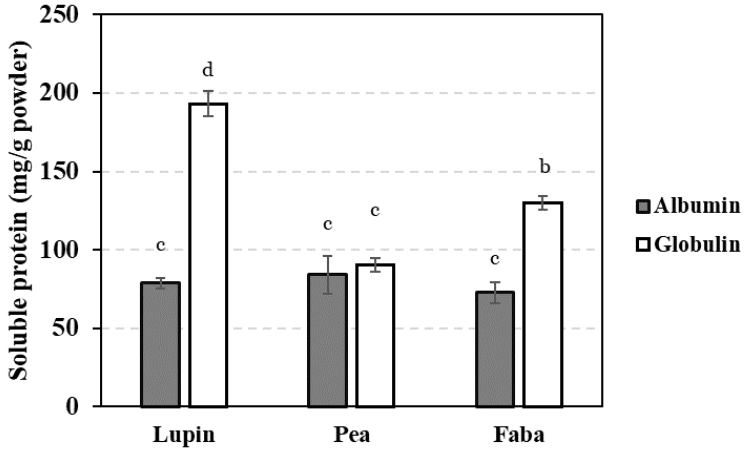
Table 5. Summary of colorimetric protein methods examined in the preliminary study

Method (detection wavelength)	**Detection limit (protein/mL sample)**	**Buffer (NaCl) compatible**	**Key characteristics**
Biuret (540 mm)	5 to 160 mg/mL	≤ 2 M	• Incubation: 37°C, 30 min • Incompatible with reducing agents, ammonium salts • Provides consistent signals independent of amino acid composition
BCA (562 nm)	0.5 to 1200 µg/mL	< 1 M	• Incubation: 37°C, 30 min • Incompatible with reducing agents, chelators, proteins rich in -SH and aromatic amino acids
Bradford (595 nm)	1 to 1500 µg/mL	< 5 M	• Incubation: RT for 10 min • Incompatible with detergents, proteins rich in Arg, His and Cys • High sample-to-sample variation

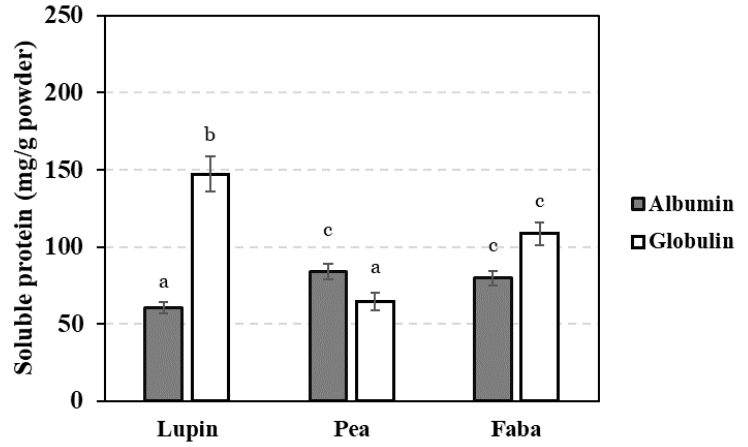
References: Layne (1957), Ninfa, Ballou, and Benore (2009); Okutucu, Dınçer, Habib, and Zıhnıoglu (2007), Pickardt et al. (2009), ThermoFisher Scientific (2019)

A relative assessment of these methods was facilitated by quantifying yield fractions from the target pulse proteins. It was found that the three methods did not produce statistically different values in most of the extracted fractions. As exhibited in **Figure 3**, the only statistical differences were found in lupin and pea globulins, where the Biuret assay showed significantly ($P < 0.05$) higher values as compared to BCA and Bradford. This may have been a result of the Biuret reagent reacting with various organic compounds that deliver intensified light scattering (Hortin & Meilinger, 2005), such as traces of starch that would have been present. The BCA method, while also possibly prone to such interference, may be less accurate due to its secondary detection of the BCA-Cu^{+} chromophore. The Bradford method was found to be a standardised option for the target samples, as it often displayed values intermediate between the BCA and Biuret methods. However, the assay does not detect proteins below 3 kDa (Silvério et al., 2012). This may be an issue when quantifying the soluble peptides released during the subsequent digestion studies, as most food-derived element-binding peptides that enhance bioavailability have been reported to be < 5 kDa (Sun et al., 2020). The quantity of peptides/proteins released during simulated digestion may also be considerably lower than the crude extracts, making the Biuret assay's lower sensitivity a potential concern. The BCA assay was thus selected for soluble protein quantification in the following studies, as used among other protein methods for validation (e.g. LECO from Laboratory Equipment Corporation for total nitrogen).

a)



b)



c)

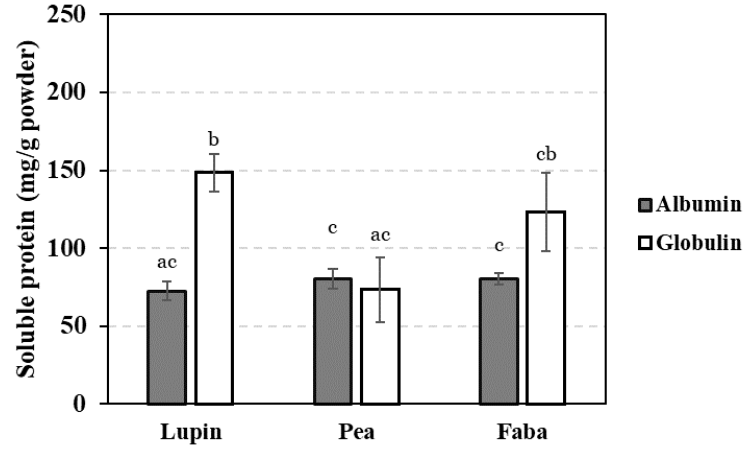


Figure 3. Soluble protein analyses of sequentially fractionated legume proteins, using a) Biuret, b) BCA and c) Bradford assays, expressed as mg/g of the original dry powder used for extraction (means ± SE, n = 3). Statistical significance is represented by difference in subscript letters in each graph ($P < 0.05$).

It has been noted that extracts from the current study showed relatively high proportions of albumin to globulin relative to the total soluble proteins, irrespective of the protein assay used (**Table 6**). This could have been confounded by cross-contamination, as SDS-PAGE studies regularly report the cross-contamination of globulins (vicilin and legumin) and other polypeptides in isolates of lupin and pea albumin (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008; Koyoro & Powers, 1987; Rubio et al., 2014). However, such difference in ratio may also stem from extractability of the proteins itself. For example, the major globulins in lupin (Conglutins α and β) are often poorly extracted in weak salt solutions (~0.4 M, versus 0.5 M used in this study) (Sironi, Sessa, & Duranti, 2005). As such, proteins that were not solubilised would not have been measured by these assays. The subsequent section thus focuses on the optimisation of globulin extraction, as the major protein fraction found in pulses.

Table 6. A comparison in the relative fraction of major storage proteins from the current study versus reported literature values. The range of values represent each fraction as a proportion of total soluble protein as examined by the three methods.

Pulse type	Fractions (%)			
	Albumins		**Globulins**	
	Current	*Literature*	*Current*	*Literature*
Lupin	21.89-23.96	5-10	54.65-55.3	79-83
Pea	24.26-33.62	21.3-26.4	20.69-25.06	55.3-80
Faba	22.32-31.56	20	31.87-36.07	60

References: Duranti et al. (2008), Estevinho and Rocha (2018), Gueguen and Cerletti (1994), Müntz, Horstmann, and Schlesier (1999), Sironi et al. (2005), Warsame, O'Sullivan, and Tosi (2018)

3.1.2. Optimisation of extraction conditions

3.1.2.1. Salt concentration for globulin extraction

Various NaCl concentrations (0.25, 0.5 and 1 M) were used in the extraction of each pulse protein to compare their differences on the soluble protein yield using the BCA method. As exhibited in **Table 7**, the optimal concentration for extraction was determined to be 0.5 M of NaCl for lupin and 1 M for faba, where these respective concentrations were employed in the subsequent digestion studies. No statistically significant differences ($P > 0.05$) can be found with pea globulins extracted using 0.5 and 1 M NaCl. 1 M NaCl was selected for pea protein globulin extraction in the later reported studies.

Table 7. Results of average globulin proteins (mg/g dry powder) extracted using three concentrations of NaCl (n = 16, means ± standard deviation)

Pulse type	Salt Concentration		
	0.25 M	**0.5 M**	**1 M**
Lupin	98 ± 5.2^{a}	148 ± 9.0^{b}	114 ± 10^{a}
Pea	36 ± 9.0^{a}	95 ± 12^{b}	99 ± 14^{b}
Faba	83 ± 7.5^{a}	152 ± 23^{b}	199 ± 7^{c}

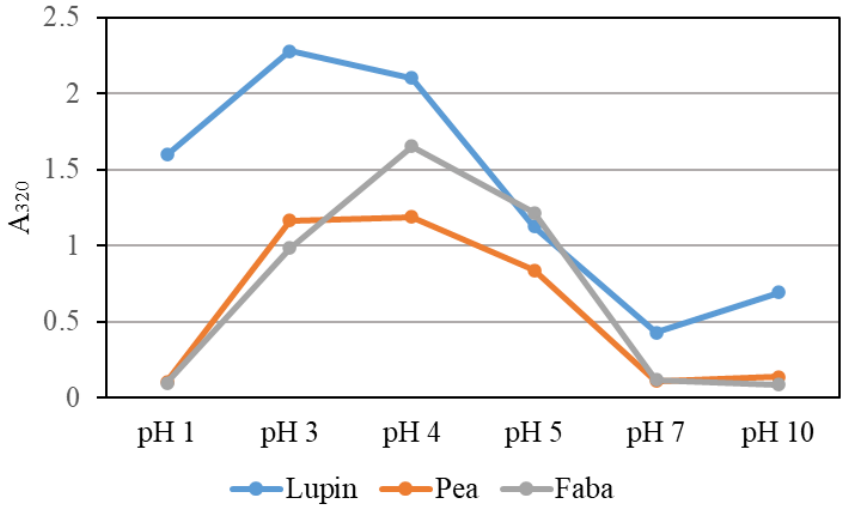
Means that share a subscript letter in each row are not significantly different ($P < 0.05$).

3.1.2.2. Isoelectric-point determination

To increase protein concentration of the extract, the fractionated proteins were filtered through a 30 μm cellulose membrane and isoelectrically precipitated. **Figure 4** demonstrates the absorbance of filtered protein fractions between pH range 2-10 when spectrophotometrically read at 320 nm. The pH was adjusted by diluting 5 mL of each supernatant using 0.5 M NaOH or HCl (Makeri et al., 2017). The isoelectric points were

determined as the points with the highest turbidity and used for the first research study (Chapter 4).

a)



b)

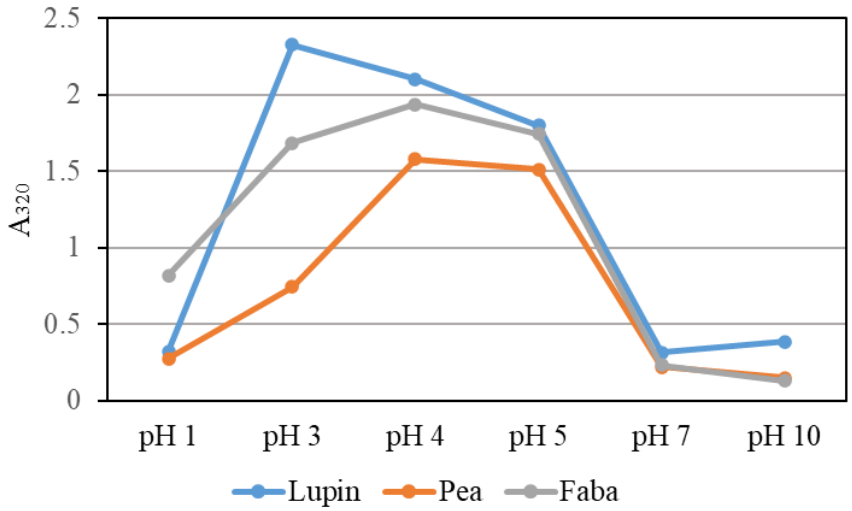


Figure 4. Solubility curves of a) albumin and b) globulins of lupin, pea and faba seed proteins as obtained by the determination of their turbidity at 320 nm. Values represent the means of triplicates (n = 3).

3.1.3. Desalting and anti-nutrient analyses

As alluded to in the Chapter 2 review, the functional properties of proteins and their derivatives are influenced by their propensity to form complexes with other molecules within the surrounding milieu. This includes other organic factors present from the pulse seed, or molecules from endogenous digestive fluids. Those that interfere with the solubility and cation-exchange capacity of the proteins would require reduction or elimination from the protein fractions. The fractions undergoing the *in vitro* digestion and metal chelating assay were thus desalted *via* dialysis. Potential anti-nutritional factors (phytic acid and total phenolics) were quantified as a reference for the digestion studies.

3.1.3.1. Phytic acid

Phytic acid is a polyanionic molecule that forms insoluble complexes with both cations and proteins. It is known to diffuse into water (Huma, Anjum, Sehar, Issa Khan, & Hussain, 2008), as well as some solvents used in Osborne extraction (Han, 1988; Martínez-Maqueda, Hernández-Ledesma, Amigo, Miralles, & Gómez-Ruiz, 2013). The desalting process would theoretically remove phytate residues as its MW is lower than the cut-off of most membrane filters (Phytate MW: 660.04 Da, common membranes: 1-20 kDa). However, phytates in pulses primarily accumulates in protein storage vacuoles as globoids (Bohn, Meyer, & Rasmussen, 2008), making separation from the protein difficult. Any remnants of phytate in the isolated pulse protein fractions can insolubilise proteins and decrease their element-binding capacity (Urbano et al., 2000). As such, phytate quantitation prior and following desalting was conducted in this preliminary study.

To validate the phytate extraction and analytical methods used, the phytate-phosphorous in each pulse (dehulled ground seed) was determined and compared to the total phosphorous as measured by ICP-OES. The proportion of phytate to total phosphorous was compared against literature values as benchmarks. As shown in **Table 8**, the method was found to produce phytate values that align with those reported in the literature.

Table 8. Range of phytic acid values obtained for the pulses investigated, as a fraction of the total P based on values determined by ICP-OES (means ± SD, n = 3).

Legume	Current phytate mg g^{-1} (as % of total P in parenthesis)	Literature Range (mg g^{-1} phytate P)	Literature reported median	Literature reported % of total P
Field pea (*Pisum sativum)*[1,2,3,4,5,6,7]	1.7 ± 0.08 (48%)	1.4 – 2.5	2.3	45 – 58
Faba bean (*Vicia faba)*[4,6,8]	2.4 ± 0.15 (60%)	0.95 – 2.4	1.5	40 – 60
Dehulled lupin (*L. angustifolius)*[2,4,5,9]	3.2 ± 0.12 (69%)	0.5 – 3.5	1.9	44 – 63

References: [1]Amarakoon, Thavarajah, McPhee, and Thavarajah (2012),[2]Steiner, Mosenthin, Zimmermann, Greiner, and Roth (2007),[3]Eeckhout and De Paepe (1994),[4]Selle, Walker, and Bryden (2003),[5]Humer and Zebeli (2015),[6]Abd El-Hady and Habiba (2003),[7]Wang and Daun (2004),[8]Carnovale, Lugaro, and Lombardi-Boccia (1988),[9]Glencross (2005)

The phytate content of each protein fraction prior and following dialysis was also quantified. As observed in **Figure 5**, phytate values in the undialysed extracts varied considerably, from 2.4 ± 1.5 mg of phytate-P/gram of solids in lupin globulin, to 26 ± 2.8 mg phytate-P/gram of solids in lupin albumin. Dialysis led to notable phytate reductions in lupin albumin (26 ± 2.8 to 6.6 ± 1.9 mg/g solids), pea globulin (18 ± 4.0 to 8.8 ± 1.2 mg/g solids) and faba globulin (23 ± 1.7 to 9.6 ± 1.4 mg/g solids), but the effect on the other

fractions were unclear. The lack of complete phytate elimination suggests that phytase may be necessary in future studies to enhance the protein fractions' solubility.

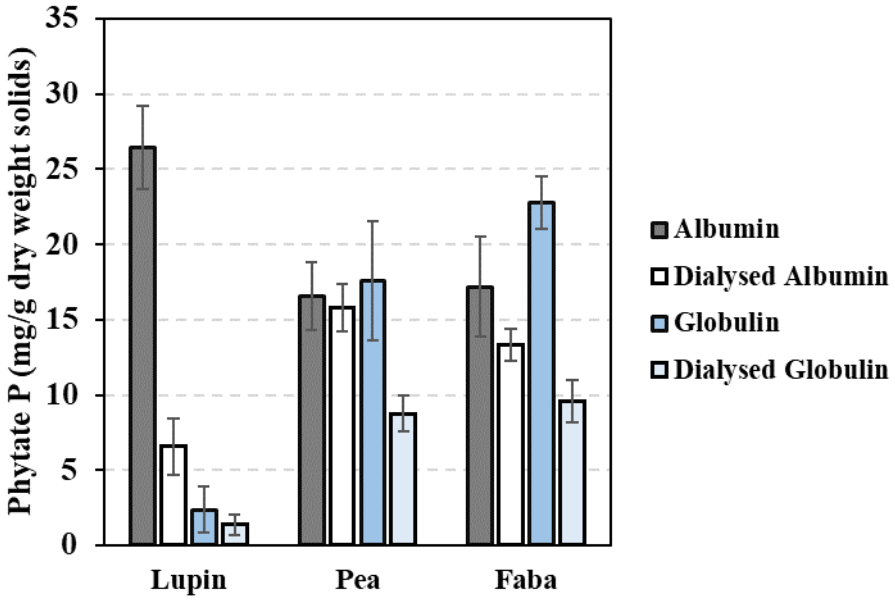


Figure 5. Phytate phosphorous in crude and dialysed (10 kDa cut-off) fractions (means ± SD, n = 3).

3.1.3.2. Phenolic compounds

Phenolic compounds are a class of organic compounds that act as ligands forming complexes with cations, which may be insoluble as alluded to in Chapter 2. They are also modulators of protein solubility, as well as their chelation capacity due to competition for amino acid residues that may chelate elements (Jakobek, 2015). Phenolic compounds in pulse cotyledons are present in both soluble and bound forms (Liu & Tang, 2015). Whilst the soluble fractions are easily extracted by water due to their hydrophilicity (Huang et al., 2018; Yogesh, Jha, & Ahmad, 2014), cell wall disruption through milling allows some further extraction of insoluble fractions (Shibuya, 1984).

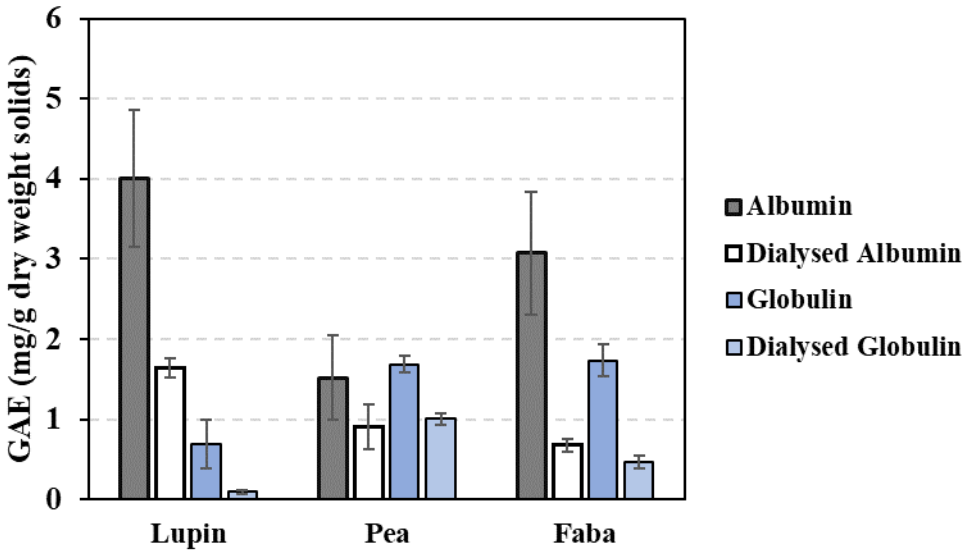


Figure 6. Total phenolic compounds reported as gallic acid equivalents (GAE) in crude and dialysed (10 kDa cut-off) fractions (means ± SD, n = 3).

The total phenolic content of each extracted pulse fraction was determined using the colorimetric Folin–Ciocalteu (FC) assay, the most routinely applied method in food analysis (Prior, Wu, & Schaich, 2005). As observed in Figure 6, dialysis was effective at reducing polyphenol concentrations to below 2 mg gallic acid equivalents per gram of extracted dry sample. The low concentration suggests that the phenolic compounds present may be unlikely to interfere with effective solubilisation of the protein fractions during the digestion study.

3.1.4. Enzymatic treatment of protein fractions

It has been established that certain classes of legume proteins can possess some resistance to proteolysis (Carbonaro, Maselli, & Nucara, 2015). Some seed storage proteins possess oligomeric structural features associated with hydrophobicity, which would inhibit

instead of solubilise elements if the element-peptide complex precipitates during digestion. For example, certain high molecular weight digestion products of 7S soy globulins are known to inhibit Fe bioavailability in humans, which likely results from the formation of indigestible complexes (Hurrell, 2003). Another study on *V. faba* and *P. vulgaris* has also found a tendency for soluble Fe to associate with the insoluble protein fraction, in turn reducing its bioavailability in rodent trials (Carbonaro, Grant, Mattera, Aguzzi, & Pusztai, 2001).

The production of protein hydrolysates can be achieved chemically, enzymatically or through microbial fermentation. Enzymes of animal, plant, and microbial origin are well-known aids to alter the physicochemical properties of food proteins, namely through adequate hydrolysis of polypeptides into smaller peptides and amino acids. Although some intrinsic proteolysis is expected throughout the simulated digestion process in this experiment (with pepsin and pancreatin), the protein fractions examined may benefit from additional enzymatic hydrolysis to improve their solubility and element-binding capacity. The properties achieved by enzymatic hydrolysis differs amongst the various types and their form (batch versus immobilised), as well as hydrolysis conditions (time, pH, temperature, and enzyme to substrate ratio) (Martínez-Maqueda et al., 2013).

For proteases, various exo- or endo-peptidases with different specificities in amide bond cleavage can be utilised during the production process (Table 9). As identified in the Chapter two review, the common enzymes used in the literature to produce Fe, Zn and Ca-binding peptides include pepsin, alcalase, trypsin, pancreatin, papain, Protease M and

Flavourzyme. In selecting an enzyme with complementary cleavage to the digestive enzymes used in the simulated model, Protease M emerged as a prospective option for the current study. The enzyme is a protease peptidase from *Aspergillus oryzae*, with a balance of both exo- and endo-peptidase activity (Amano Enzyme Inc, 2020). It was reported by multiple authors in the review (e.g. Lv et al. (2013)) to have generated soluble Fe- or Ca-binding peptides from soy proteins, which are known to be only hydrolysed to a limited extent by trypsin alone (Tsumura, Saito, Kugimiya, & Inouye, 2004).

Table 9. Properties of proteases used in food processing, adapted from Belitz et al. (2009)

Type	**Source**	**Enzyme/Origin**	**Notes**
Serine Endopeptidases	Animal	Trypsin Chymotrypsin Elastase Plasmin	- Ser and His residue in their active sites - Alkaline proteinases - Activity confined to pH range 7 – 11.
	Microbial	*Bacillus spp.* *Streptomyces spp.*	
	Fungal	*Aspergillus spp.* *Tritirachium spp.*	
Cysteine Endopeptidases	Plant	Papain Bromelain Ficin	- Cys residue in active site - Sensitive to oxidising agents - Activity range pH 4.5 – 10, maximum 6 – 7.5
	Microbial	*Streptococcus proteinase*	- Can be deactivated using oxidative/alkylating agents, metal ions
Metallopeptidases	Microbial	*Bacillus spp.* *Streptomyces grises*	- Most contain a metal ion in active site
	Fungi	*Aspergillus niger*	- Active in pH range 6 – 9 - Low specificity - Inhibited by chelators/SDS
Aspartic Endopeptidases	Animal	*Pepsin* *Rennin* *Cathepsin D*	- Two aspartic residues in the active site - Active in pH range 2 – 7
	Fungi	*Aspergillus spp.* *Penicillium spp.* *Trametes sanguinea* *Mucor spp.*	

Based on findings from the preliminary experiment (section 3.1.3.), the addition of phytase is likely needed to reduce residual phytic acid in the extracted protein fractions. Whilst proteases hydrolyse polypeptides into smaller peptides and amino acids, phytase dephosphorylates the 6 phosphate groups on the myo-inositol ring of phytate in a step-wise manner. This decreases the chelation capacity of phytic acid in binding minerals, and also improves the susceptibility of the protein bodies to further enzymatic action by protease (Tous et al., 2021).

Phytase has been isolated from a range of sources, all of which have a near neutral optimum pH and temperature range between 40 and 80 °C (**Table 10**). These are generally classified depending on the carbon molecule in which hydrolysis is initiated, although the degree of dephosphorylation is of greater pertinence to this study than the hydrolysis location. Considering that phytate is embedded in the protein bodies of pulses, simultaneous hydrolysis using protease and phytase may be desirable to enhance accessibility of both enzymes to their substrates. This can be achieved by the addition of the two enzymes together or sequentially into the sample extract, or by using microorganisms that express both proteases and phytases. Based on previous findings from the literature, *Lactobacillus plantarum* has been found to be particularly effective at phytate degradation in legume matrices (De Pasquale, Pontonio, Gobbetti, & Rizzello, 2020; Mohamed, Abou-Arab, Gibriel, Rasmy, & Salem, 2011). *L. plantarum* spp. are also known to possess proteolytic enzymes that hydrolyse other pulse proteins, such as *Phaseolus vulgaris* (Rui et al., 2015). Given this knowledge, both enzymatic hydrolysis and fermentation using *L. plantarum* strains are feasible options to explore in the bioprocessing chapter.

Table 10. Kinetic properties of phytases from various sources

Source	Type	Optimum pH	Optimum Temperature (°C)
Plant	Faba bean	5	50
	Lupin seeds	5	50
	Mung beans	7.5	57
	Navy beans	5.3	50
	Soybean	4.5 – 5	55 – 58
	Wheat	5 – 5.5	50 – 60
Fungi	*Aspergillus spp.*	5 – 6	50 – 70
	Penicillium simplicissimum	4	55
Microbial	*Bacillus spp.*	6.5 – 8	55 – 70
	Klebsiella spp.	4.5 – 5.5	50 – 68
	Escherichia coli	4.5	55 – 60
	Lactobacillus sanfranciscensis	4	50
Yeast	*Pichia spp.*	3.6 – 5.5	70 – 80
	Candida spp.	4.5 – 4.6	40 – 65

Sources: Brejnholt, Dionisio, Glitsoe, Skov, and Brinch-Pedersen (2011), Kaur, Kunze, and Satyanarayana (2007), Kumar and Sinha (2018), Tazisong, Senwo, Taylor, and He (2008)

3.1.5. Protocols for preliminary experiments

The sources of all reagents used for the preliminary studies can be found in **Table 11**. All experiments were performed in triplicate, using Milli-Q water (⩽18 MΩ) produced using a Synergy UV Millipore System (Merck Millipore, Australia). The centrifuge used was Eppendorf 5810R (Sigma-Aldrich, Castle Hill, New South Wales, Australia). All spectrophotometric measurements were made on Multiskan™ GO (Thermo-Fisher Scientific, Scoresby, Victoria, Australia).

Table 11. List of chemical reagents used in the preliminary studies

Supplier	Reagents
Chem-Supply	*n*-hexane, Sodium Chloride (NaCl), Sodium hydroxide (NaOH), Potassium hydroxide (KOH), Hydrocholoric acid (HCl), Phosphoric acid (H_3PO_4)
Sigma-Aldrich	Copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), Potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$), Potassium iodide (KI), ethanol absolute, methanol, Calcium chloride ($CaCl_2$), Cetrimonium bromide (CTAB), Sodium borate ($Na_2[B_4O_7] \cdot 10H_2O$), Sodium phytate, Folin-Ciocalteu (50%), gallic acid, Sodium Carbonate (Na_2CO_3), Bovine serum albumin (BSA)
Bio-Rad	Coomassie Brilliant Blue G-250
Spectrum Chemicals	Glyoxalbis(2-hydroxyaniline)
Thermo-Fisher	Pierce BCA assay kit

3.1.5.1. Protein fractionation

Ground and dehulled cotyledons of the field pea (*Pisum sativum L.*), faba bean (*Vicia faba major*) and sweet lupin (*Lupinus angustifolius L.*) were provided by Dr. Joe Panozzo and Dr. Jason Brand at Agriculture Victoria. The ground powders were defatted by vigorous stirring with *n*-hexane (1:5, w/v) for 3 h at room temperature (RT) and centrifuged at 2465 *x g* for 10 min at 10°C. The pellets were washed with 0.1 M KOH to neutralise acidic components of plant cell tissues in assisting protein extraction for the subsequent steps. The pellets were then dehydrated under the fume hood, and stored at 4°C until used for extraction.

The water- and salt-soluble (albumin and globulin) fractions were sequentially extracted following the method of Makeri, Mohamed, Karim, Ramakrishnan, and Muhammad (2017). Defatted powders were suspended in Milli-Q 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 *x g* for 20 min at 10°C. The supernatant was decanted as the albumin fraction, and the residue were initially extracted with 0.5 M NaCl solution (1:6, w/v) for 2 h to yield the globulin proteins for all pulses. To determine the ideal salt concentration for the extraction of globulins for each pulse, the defatted meal was suspended in NaCl (1:6, w/v) at various molar concentrations (0.25 M, 0.5 M and 1.0 M). The globulins were extracted and centrifuged under the same conditions described above, with the soluble protein in the supernatant measured as described in section **3.1.5.2.** below.

The supernatants of each pulse fraction were dialysed at room temperature in MilliQ water (RT, 21°C) for 4 h followed by stirring overnight at 4°C, using tubings with 12 kDa cut-off (D-9652, Sigma-Aldrich, Bayswater, Victoria, Australia). Following dialysis, samples were filtered using a cellulose membrane (10311897, Whatman, Thermo-Fisher Scientific, Scoresby, Victoria, Australia) to remove insoluble starch formed during dialysis. Each fraction was then pooled and precipitated at their isoelectric points as determined in section **3.1.5.1.1.** below. A final centrifugation (2465 *x g*, 5 min) was conducted to yield the precipitated proteins. They were then freeze-dried overnight and stored at 4°C until analysis.

3.1.5.1.1. *Isoelectric-point determination for precipitation*

To determine the isoelectric point used to precipitate the proteins in the extracted fraction, 5 mL of each supernatant was diluted by adjusting to pH values between 2.0 and 10.0 using 0.5 M NaOH or HCl (Makeri et al., 2017). The turbidity of each supernatant was

read at 320 nm using the 96-well microplate reader noted in the introduction of **3.1.5**. The pH exhibiting maximum absorbance was considered as the isoelectric point of that fraction and used for subsequent extraction and analyses.

3.1.5.2. Soluble protein determination

The methods used to determine soluble protein content included the Biuret method, the Bradford method and the Bicinchoninic acid method (BCA). These assays were adapted for analysis based on colour intensity development between the sample and reagent at 10 min (RT for Bradford) or 30 min (RT for Biuret, 37°C for BCA), using the 96-well microplate reader mentioned above. The measurements took place using a sample:reagent ratio of 1:5 for the Biuret assay (read at 540 nm), 1:50 for Bradford (read at 595 nm) and 1:7 for BCA (read at 562 nm). Bovine serum albumin (BSA) was used for all standard curve preparation. The BCA method was conducted following a commercial kit (Pierce BCA Protein Assay Kit, Thermo-Fisher Scientific).

3.1.5.2.1. Biuret method

The Biuret assay was performed according to the method described by Nowotny (1979). The reagent was prepared by dissolving 1.5 g of $CuSO_4 \cdot 5H_2O$ and 4.5 g of $KNaC_4H_4O_6 \cdot 4H_2O$ in 250 mL of 0.2 M NaOH solution, followed by the addition of 2.5 g of KI. The resulting solution was brought to a total volume of 500 mL as the final reagent.

3.1.5.2.2. Bradford Method

The Bradford assay was performed based on the original method described by Bradford (1976). The reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue

G-250 in 50 mL of 95% ethanol. The solution was carefully mixed with 100 mL of 85% H_3PO_4 and filtered using a cellulose membrane (10311897, Whatman, Thermo-Fisher Scientific) before bringing the total volume to 1 L with MilliQ water. The reagent was stored in an amber glass bottle and kept at 4 °C, which was brought to RT before use.

3.1.5.3. Anti-nutritional compound analyses

3.1.5.3.1 Phytic acid analysis

The samples were prepared for phytate analysis according to Gao et al. (2007). In summary, 0.5 g of solid 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 *x 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 *x g* for 20 minutes to dissolve the salt. The mixture was allowed to settle at -20°C for 20 min, before being centrifuged at 2465 *x g* at 10°C for 20 min.

Spectrophotometric analysis of total phytic acid (PA) were performed using the method reported by Agostinho et al. (2016) using the 96-well microplate reader noted above. In summary, 76 µL of $CaCl_2$ solution (10 mg/L), 15.2 µL of glyoxalbis(2-hydroxyaniline) in methanol (1 g/L), 76 µL of ethanol/methanol mixture (70:30 v/v), 137 µL of borate buffer solution (pH 12.5) containing NaOH (5 g/L), $Na_2B_4O_7 \cdot 10H_2O$ (5g/L) and CTAB (1 mmol) are combined and added to each well containing 76 µL of sample or phytic acid as a standard. The analytical blank solution was prepared by replacing the PA

solution with water, with a six-point calibration curve is prepared from a sodium phytate stock solution (200 mg/L). The absorbance of the mixture was measured at 500 nm.

3.1.5.3.2. Total phenolics assay

The quantitative of total phenolic compounds for each fraction was determined by the Folin-Ciocalteu spectrophotometric method (Lin & Tang, 2007) adapted for analyses using the 96-well microplate reader noted above. In summary, an aliquot of the sample or standard solution (20 µL) is mixed with 100 µL of 50% Folin-Ciocalteu reagent (previously diluted to 1:10 in distilled water) and 80 µL of 2% Na_2CO_3. Absorbance was measured after 30 min of incubation at room temperature at 750 nm. The standard curve was constructed using gallic acid as the standard, and the final phenolic content expressed as gallic acid equivalents (mg GAE g^{-1} of dry mass).

3.2. Analysis of metal-binding capacity

Existing methods proposed to examine elemental-binding capacity primarily involve combining the specific protein hydrolysate with a saturated solution of established complexing agents (**Table 12**). As previously suggested, a standardised method for endpoint detection and expression would be ideal in this study. This can be facilitated using the solubility-centrifugation method, coupled with a sensitive endpoint detection method such as the inductively-coupled plasma optical emission spectrometry (ICP-OES).

Table 12. Various literature methods used in the assessment of Fe, Zn and Ca-binding

Element	Binding agent	Basic assay method	Endpoint detection of unbound elements	Reference
Fe	$FeCl_2$, $FeCl_3$, $FeSO_4$, $(NH_4)2Fe(SO_4)_2$	- Colorimetric (Fe^{2+}-ferrozine at 562 nm, Fe^{2+}-2,2'-bipyridine at 522 nm) - Titration (EDTA-xylenol orange or Cobalt (II) Sulfate) - Solubility: metal-peptide solutions shaken at RT (2h), centrifugation	- Colorimetric: *ortho*-phenanthroline reagent - Titration: colour change (xylenol orange: purple to yellow, Cobalt (II) Sulfate: blue to light pink) - Solubility: Atomic Absorption Spectrophotometry (AAS)	Carter, (1971); Eckert et al., (2014); Eckert et al., (2016); Zhou, Sun, & Canning, (2012)
Zn	$Zn(O_2CCH_3)_2$, Zn ion, $ZnSO_4$	- Precipitation (zinc ion-triethylamine) - Titration (EDTA-xylenol orange-hexamethylenetetramine) - Solubility: metal-peptide solutions shaken at RT (2h), centrifugation	- Precipitation and solubility: Atomic Absorption Spectrophotometry (AAS) - Titration: colour change (xylenol orange and: purple to yellow)	Eckert et al., (2014); Wang et al., (2012; Wang, Zhou, Tong, & Mao, (2011)

The optimal pH conditions for assaying various Fe, Ca and Zn salts are based on their solubility, which has been previously determined for $FeCl_2$/$FeCl_3$, $CaCl_2$, and $Zn(O_2CCH_3)_2$ by Eckert et al. (2014) to be pH 7 for Fe and pH 8 for Ca and Zn. The same mineral salts will thus be utilised in this study, although $ZnCl_2$ is used due to its higher solubility (National Center for Biotechnology Information, 2019).

3.3. Simulated digestion and bioaccessibility

A medley of *in vivo* and *in vitro* methods are currently exploited in the literature to examine nutritional bioavailability/bioaccessibility (Cardoso, Afonso, Lourenço, Costa, & Nunes, 2015). Whilst the advantages provided by true physiological contexts of *in vivo* studies are important for validating any effects from *in vitro* studies, the introduction of host-related factors can introduce ambiguities in understanding bioaccessibility. An *in vitro* model that includes the principal processes of upper gastrointestinal digestion (predominantly gastric, small intestinal) and colonic fermentation would enable systematic identification of the changes in peptide properties and element solubility with good experimental control. These can be either static or dynamic in nature. Dynamic models enable real-time regulation of pH, flow and enzyme addition during each compartment of digestion, whereas static models are designed with a constant ratio of enzymes and fluids (Brodkorb et al., 2019).

Most investigations that have applied an *in vitro* model to evaluate element-peptide complexes have simulated the two key steps of gastric and intestinal digestion through static models, followed by assessments of fractional solubility (Chen et al. (2013), dialysability (Wang et al., 2011), or uptake by Caco-2 cell cultures (Eckert et al., 2016). However, the saliva released within the oral cavity is a source of endogenous mineral salts and proteins, both of which may influence the solubility of elements (Buzalaf, Hannas, & Kato, 2012; Delimont, Rosenkranz, et al., 2017). It would thus be valuable to incorporate the oral step into the digestion model within the current study, and consider the representative concentrations of these factors in the simulated salivary fluid.

Given the hydrophobic nature of some pulse proteins, it is also expected that some proteins or peptides that chelate elements would not dissociate (i.e. become soluble) during small intestinal digestion. The fate of these undigested peptides and elements is important to understand, as they may mediate health effects *via* the gut microbiome. For example, if peptides containing Fe disintegrate during colonic fermentation, the release of free Fe ions may instigate the production free radicals through Fenton and Haber-Weiss reactions that cause mucosal damage and acute inflammation (Kamdi & Palkar, 2015), or stimulate the growth of pathogenic bacteria (Rusu et al., 2020). On the other hand, free Zn has the potential to be absorbed through various mechanisms (Christakos, Dhawan, Porta, Mady, & Seth, 2011; Gopalsamy et al., 2015).

The initial study will examine the *in vitro* solubility of elements as digested under both fasted and fed conditions, as characterised by different bile concentrations and transit time. These factors may affect the extent of protein digestibility, which is a critical determinant of whether the resulting complex is soluble and/or bioaccessible. The element-binding activity will be quantified for experimental runs emphasising individual steps of digestion, where each run will have an endpoint of either gastric or intestinal digestion. Elemental analysis will be conducted on all fractions of digestion in order to illustrate bioaccessibility at each digestion step. This will permit examination of the changes in element-binding activity of the peptide as it travels through the simulated gastrointestinal tract. After the candidate fraction has been selected and bioprocessed, the effects of colonic fermentation will be examined in the final study examining biomarkers of health.

3.3.1. Measurement of protein hydrolysis

The degree of protein hydrolysis is a useful marker in the extent of proteolysis and peptide size, through measuring the percentage of cleaved peptide bonds (Walters et al., 2018). This information may be useful in the initial stages of the proposed study to evaluate changes in protein fractions during simulated gastrointestinal digestion. Previous studies on Zn-chelating peptides have shown that up to a certain reaction time, a high correlation between changes in the degree of hydrolysis (DH) and binding capacity can be observed, depending on the enzyme used (Chen et al., 2013; Wang et al., 2011). According to Morais et al. (2013), the methods to examine DH are based on one of the following principles, which occur following peptide hydrolysis; 1) soluble nitrogen using a precipitation agent, 2) % of free α-amino groups, 3) the protons released from peptide bonds, and 4) changes in protein freezing point.

For the current study, a method that measures the % of free α-amino groups may be an active indicator in the availability of side chain functional groups for binding (either to elements or water). An efficient method employed in the literature utilises the reaction of α-amino groups with *o*-phthaldialdehyde (OPA), which forms a coloured compound detectable at 340 nm in the presence of a reducing agent (Nielsen, Petersen, & Dambmann, 2001). The DH is determined using Serine as a standard, as its reaction displays a response approximating the average of different amino acids. As such, the OPA method is utilised for measuring protein hydrolysis in this study.

Chapter 4. Screening for suitable pulse fractions as candidates for Fe/Zn bioaccessibility enhancement

This scoping study aimed to investigate the effects of water- and salt-extractable proteins (albumin and globulin) from pea, lupin and faba beans on the efficacy of Fe and Zn fortification. Dosages based on current Australian dietary recommendations were selected, and the solubility of these elements were measured following *in vitro* digestion as a proxy for bioaccessibility. The necessity of this study stems from the intrinsic presence of anti-nutritional factors from pulses, which is a primary barrier to their utility as soluble element carriers. Namely, the digestion-resistant proteins, certain polyphenols and phytic acid may hinder the capacity of legume proteins as bioaccessible element delivery agents. Outcomes of this research provided fundamental insight towards the selection of the optimal legume fraction for further bioprocessing.

This chapter contains the first research study as published in *Current Research in Food Science*. The version included has been peer-reviewed without editorial copyediting, typesetting and proofreading. The full reference can be found in the preface of the thesis.

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.

4.1. 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, Salam, Kumar, & Bhutta, 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, Blair, & Glahn, 2011; Tako, Reed, Anandaraman, Beebe, Hart, & Glahn, 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 (PA) contents, both of which are co-associated in

protein storage vacuoles of the cotyledon *in planta* (Raes, Knockaert, Struijs, & Van Camp, 2014). Legume proteins consist predominantly of peptides with multimeric structure and arranged in the β-sheet conformation (Carbonaro, Maselli, & Nucara, 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, Freitas, & Teixeira, 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 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, Stockmann, Ng, & Ajlouni, 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, De Stefano, Milea, & Sammartano, 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, Yupanqui, Sirinupong, Alashi, Aluko, & Youravong, 2018; Fu et al., 2020) and chickpea (Torres-Fuentes, Alaiz, & Vioque, 2012)). However, some hydrolysates may also participate in the formation of insoluble ternary complexes with PA through a cationic bridge under physiological pH (Zhang, Stockmann, Ng, & Ajlouni, 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, Dassenko, Cook, Juillerat, & Hurrell, 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, Ruggeri, Aguzzi, & Cappelloni, 2003; Lombardi-Boccia, Schlemmer, Cappelloni, & Lullo, 1998).

PA levels in legumes can be reduced with common domestic food processing approaches such as soaking or fermenation, although the extent of reduction can be small (Raes,

Knockaert, Struijs, & Van Camp, 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.

4.2. Materials & Methods

4.2.1. General reagents and equipment

Glyoxal bis(2-hydroxyaniline) was acquired from Spectrum Chemicals (Gymea, New South Wales, 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 *a*-amylase (from *Aspergillus oryzae*) were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Pepsin and pancreatin were acquired from Thermo-Fisher Scientific (Scoresby, Victoria, Australia). All other chemicals and organic solvents used were of analytical-grade or better. Deionised water ($\leqslant$18 MΩ) used was produced using a Synergy UV Millipore System (Merck Millipore, Victoria, Australia).

All UV-Visible spectrophotometric measurements were made on Multiskan™ GO from Thermo-Fisher Scientific (Scoresby, Victoria, 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, Victoria, Australia).

4.2.2. 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 *x 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, Mohamed, Karim, Ramakrishnan, & Muhammad, 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 *x 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, Pérez, Ruiz, Guzmán, Aranda‐Olmedo, & Clemente, 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 %.

4.2.3. 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, Shang, Maroof, Biyashev, Grabau, Kwanyuen, 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 *x 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 *x g* for 20 minutes to dissolve the salt. The mixture was allowed to settle at -20°C for 20 min, before being centrifuged at 2465 *x 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, de Souza Oliveira, Anunciação, and Santos (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 (5g/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 minutes 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.

4.2.4. Total phenolics 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 ^oC. 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).

4.2.5. Mineral chelation

4.2.5.1. 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, Bamdad, and Chen (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).

4.2.5.2. 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 (produced when salivary glands are

activated through sensory stimulation and mastication) and non-stimulated saliva (produced at rest) *in vivo* (*Supplementary Data 1*). 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, Egger, Alminger, Alvito, Assunção, Ballance, et al., 2019), whilst the currently formulated salivary fluid was utilised to account for the additional ionic salts present in natural saliva that may influence element solubility.

4.2.5.3. Simulated gastric and small intestinal digestion

A two-step digestion system adapted from our previous work (Zhang, Panozzo, Hall, & Ajlouni, 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 *x g*. Digestion was ceased by adjusting sample pH to 6.8 with 1 M NaOH. Digestion triplicates were subjected to centrifugation at 2465 *x g* for 10 mins 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 - 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 *x g*, and digestion was terminated by placing samples in ice prior to centrifugation at 2465 *x g* for 10 minutes. 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.

4.2.6. 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, Petersen & Dambmann (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.

4.2.7. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

4.2.7.1. Acid digestion

All samples were digested according to our previous work (Zhang, Panozzo, Hall, & Ajlouni, 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.

4.2.7.2. ICP-OES Specifications

ICP analyses were performed using a radial view Optima 8300 DV ICP-OES (Perkin Elmer, Glen Waverley, Australia). Samples were injected using an automated sampler (S10, Perkin Elmer, 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.

4.2.8. 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

4.3. Results

4.3.1. Proximate and anti-nutritional compound 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, Walker, & Bryden (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 13**).

Table 13. The PA to metal molar 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, Hunt, Glahn, Welch, Menkir, & Maziya-Dixon, 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, Tomé, & Mirand, 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.

4.3.2. Element bioaccessibility during *in vitro* gastric-intestinal digestion of different legume protein fraction

4.3.2.1. 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 Figures 7 and 8 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. 7**). 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 7-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. 7-B**) or GI-fed (**Fig. 7-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. 7-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. 7-C**), when compared to the control (48 vs 13 µg).

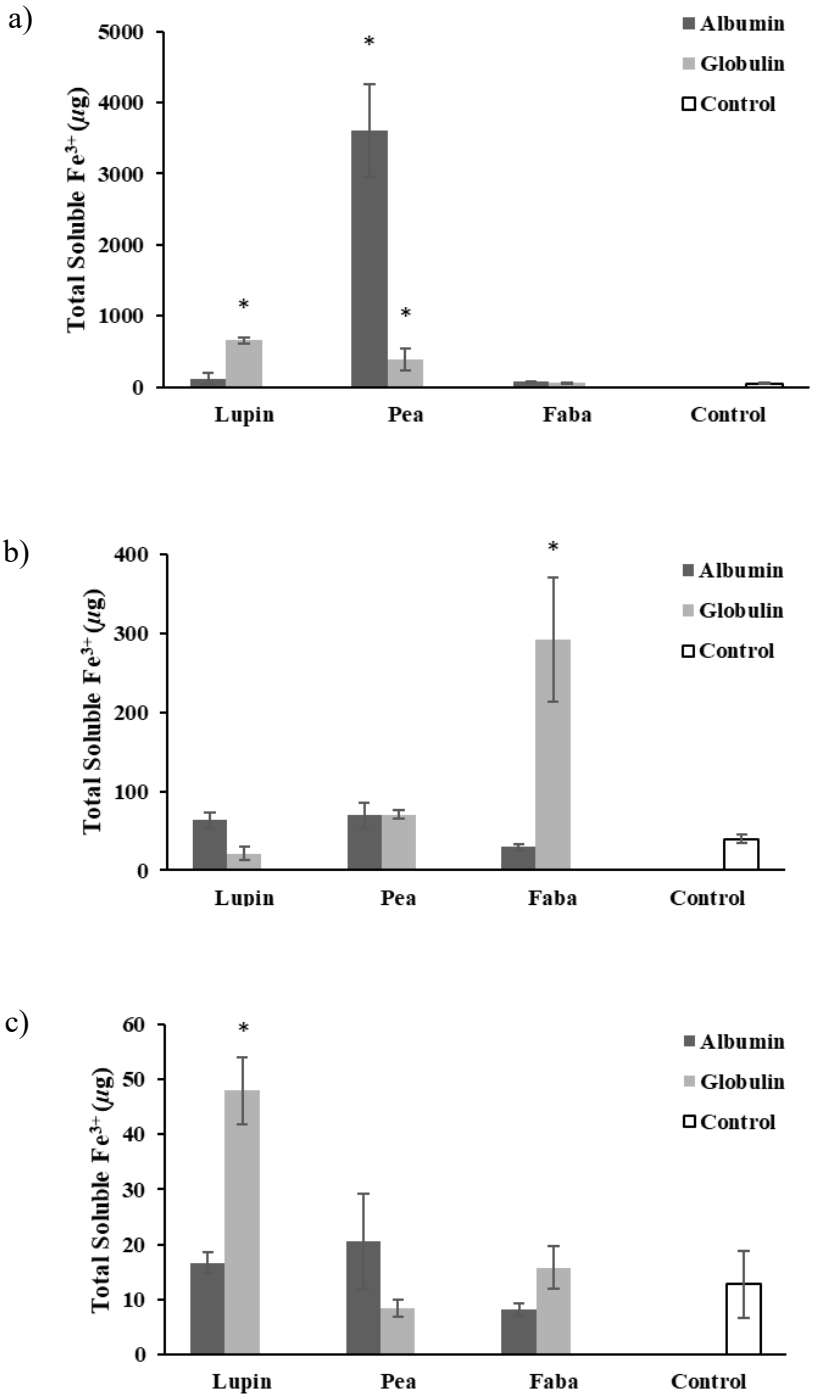

Figure 7. Soluble Ferric (Fe^{3+}) from mineral salt control and mineral fortified legume protein fractions subjected to a) oral-gastric (G), b) sequential oral-gastric-fasted intestinal (GI-Fasted), or c) sequential oral-gastric-fed intestinal (GI-Fed) stages of digestion. Columns with an asterisk (*) are significantly different from that of the control mineral salt ($P < 0.05$). Results are expressed as means ± SEM (n = 3).

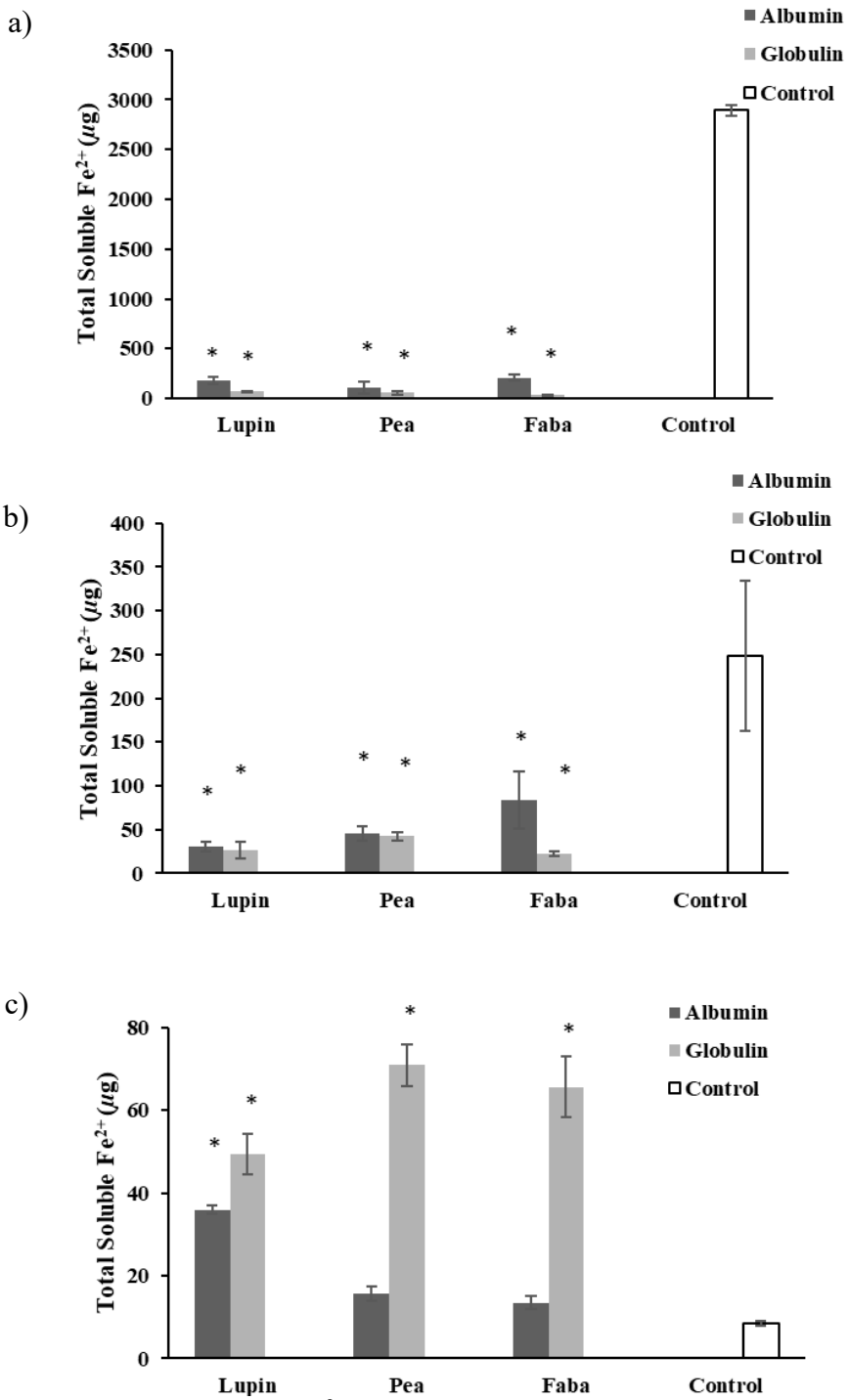

Figure 8. Soluble Ferrous (Fe^{2+}) from mineral salt control and mineral fortified legume protein fractions subjected to a) oral-gastric (G), b) sequential oral-gastric-fasted intestinal (GI-Fasted), or c) sequential oral-gastric-fed intestinal (GI-Fed) stages of digestion. Values with an asterisk (*) are statistically significantly different from that of the mineral salt control ($P < 0.05$). Results are expressed as means ± SEM ($n = 3$).

The ferrous salt showed higher solubility in the control samples (**Fig. 8-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. 8-B**) and GI-fed (**Fig. 8-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. 8-A**) and GI-fasted (**Fig. 8-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. 8-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.

4.3.2.2. Zinc

Mixed patterns of Zn solubility were observed during different digestion stages in the presence of legume protein fractions (**Figure 9**). 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. 9-A**). However, during the fasted intestinal phase (**Fig. 9-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).

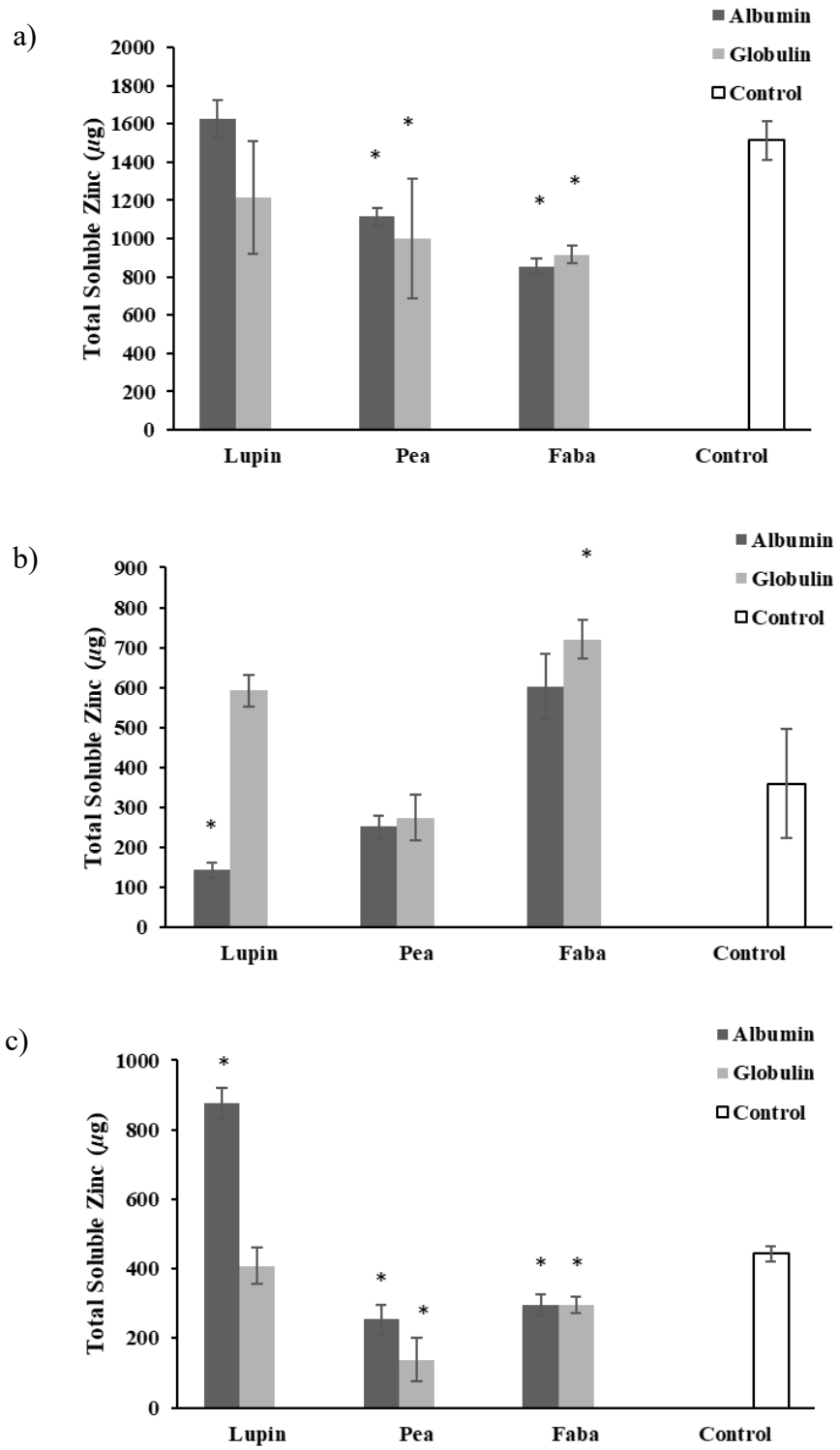

Figure 9. Soluble zinc from mineral salt control and mineral fortified legume protein fractions subjected to a) oral-gastric (G), b) sequential oral-gastric-fasted intestinal (GI-Fasted), or c) sequential oral-gastric-fed intestinal (GI-Fed) stages of digestion. Values with an asterisk (*) are statistically significantly different from that of the mineral salt control ($P < 0.05$). Results are expressed as means ± SEM ($n = 3$).

Zn solubility under the fed digestion stage (**Fig. 9-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 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.

4.3.3. Overall patterns and source of variation

4.3.3.1 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).

4.3.3.2. Zinc

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

4.3.4. Soluble protein

The results of soluble protein are presented in **Table 14**. The proteins were gradually released as soluble form into the digesta as the 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 (i.e. lupin albumin, faba albumin and faba globulin) (**Table 14**). Similarly, significantly higher levels of soluble Fe^{2+} (compared to the salt control, **Figure 8**) 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.

Table 14. 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 15. 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-NH_2 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

4.3.5. 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 increase in α-amino groups was observed in all legume protein fractions with time, suggesting effective continuation of proteolysis as digestion progressed (**Table 15**). 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.

4.4. 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 (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, Acebron, Raja, & Simpson, 2002). Meanwhile, Zn is known to bind both PA and legume oligomeric peptides with strong affinity at near small intestinal pH (~ 6.8) (Zhang, Stockmann, Ng, & Ajlouni, 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, Diepenmaat-Wolters, Zamora, 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, Santa Ana, Cole, & Fordtran, 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. 8-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, Rodriguez-Ramiro, Rodriguez-Celma, Sharp, Balk, & Fairweather-Tait, 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 solubilisation 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. 7, 8 and 9**). 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, Bai, Wang, & Zhu, 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, Vora, Hofmann, Gray, & Khosla, 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., 2003; Lombardi-Boccia et al., 1998), 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 dialysability 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 dialysability. When the authors enzymatically dephytinized the albumin fraction, this led to a significant increase in dialysability, 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, Acebron, Raja, & Simpson, 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 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.

4.5. 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 of 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 solubilized 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.

Chapter 5. Production of Fe- and Zn-binding legume protein hydrolysates through bioprocessing

The initial scoping study revealed the potential of several legume-derived protein fractions to enhance Fe and Zn bioaccessibility. However, their solubilisation efficacy was inconsistent during the major absorption site of the small intestine. This may be attributed to the residual phytic acid (PA) content of the fractions during extraction, and/or the element-binding peptide sequences being released only during extended hydrolysis times in the presence of bile (fed stage of digestion). The presence of both factors suggest that the legumes' protein solubility was limited during small intestinal digestion, and that enhancing the solubility may lead to improvements in their ability to solubilise these elements.

This chapter aims to explore the effects of some bioprocessing approaches on the solubility and metal-binding properties of a selected fraction from our preliminary screening study. Enzymatic hydrolysis using protease M, phytase, and fermentation using lactic acid bacteria have been chosen to address the residual PA levels and enhance protein solubility. Based on previous findings from the literature, *Lactobacillus plantarum* has been found to be particularly effective at phytate degradation in legume matrices (De Pasquale, Pontonio, Gobbetti, & Rizzello, 2020; Mohamed, Abou-Arab, Gibriel, Rasmy, & Salem, 2011) and will be utilised in this chapter. A protease with both endo- and exo-peptidase activity has been chosen due to its reported ability to produce calcium-binding peptides from soy proteins (Bao et al., 2008), which may also bind to other divalent cations.

In this investigation, the pea protein extract was selected for bioprocessing due to the high Fe^{3+} binding of its gastric-proteolysed products from the initial study (Chapter 4), with Zn^{2+} also examined for the same fraction to see whether any improvements in bioaccessibility can be observed. Considering that the legume albumin fractions extracted from studies in Chapter 4 likely contained a mix of both albumin and globulin proteins based on yield (Chapter 3), a pea protein concentrate was used for bioprocessing for this experiment. However, findings from Chapter 4 also suggested that faba and lupin proteins, especially globulins, may be examined in future research.

This chapter contains the second research study as published in *Journal of Agricultural and Food Chemistry*. The version included has been peer-reviewed without editorial copyediting, typesetting and proofreading, with the reference style adapted to match that of the thesis. The full reference can be found in the preface of the thesis.

Bioprocessing of Pea Protein can Enhance Fortified Fe but Reduce Zn In Vitro Bioaccessibility

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ABSTRACT:

The bioaccessibility of minerals during food digestion is essential in facilitating absorption, and hence mineral bioavailability. Bioprocessing approaches have shown promising effects on Fe and Zn bioaccessibility in plant food matrices. In this study, lactic acid bacteria fermentation or enzymatic hydrolysis were performed on pea protein concentrates (PPC) to investigate their effects on the bioaccessibility of fortified Fe and Zn salts. Simulated digestion studies revealed that enzymatic hydrolysis was more effective than fermentation. Phytase treatment significantly ($P < 0.05$) improved Fe^{3+} bioaccessibility by 5- and 12- fold during fasted and fed digestion stages, respectively. Combined phytase and protease hydrolysis led to a 6- and 15- fold enhancement of Fe^{3+} bioaccessibility in these stages. None of the bioprocessing approaches led to significant promotive effects on Zn^{2+} bioaccessibility during fasted or fed digestion. Results of this study show the potential of enzymatic treatment of PPC to significantly promote Fe bioaccessibility.

5.1. Introduction

Approximately 2 billion people suffer from a chronic deficiency of micronutrients globally, including that of essential dietary minerals such as Fe and Zn (Magee & McCann, 2019). Deficiency of Fe is the most common nutritional disorder worldwide, despite it being a critical component of metalloenzymes and haemoglobin involved in energy production and immunity (Drago, 2017). Zn also plays a critical role in metabolism, growth, immunity and reproductive functions, although its actual deficiency remains unknown due to a lack of reliable physiological biomarkers (King et al., 2015). It has been reported by Martinez-Finley, Chakraborty, Fretham, and Aschner (2012) that adequate consumption of essential cations including Fe and Zn can reduce the cellular uptake of toxic heavy metals such as Cd, Pb and Hg within the human body.

Bioaccessibility requires that a compound be released from the carrier matrix during digestion and thereby is made available for intestinal absorption (Rein et al., 2013). The fraction of elemental species that are bioaccessible tend to exist in solution or on the surface of particulates at the absorption site (Apostoli, 2006). This behaviour is determined by the elements' oxidation state, solubility, and the dynamic equilibria between the different complexing ligands present during digestion. In the absence of a soluble ligand such as protein, dissociated Fe and Zn salts can easily form insoluble precipitates with components from both digestive fluids (e.g. phosphates and oxides) or the carrier matrix (e.g. phytate) constraining their bioaccessibility.

Bioprocessing methods are conventionally recognised to enhance the bioavailability of non-heme Fe and Zn in plant-based foods (Nkhata, Ayua, Kamau, & Shingiro, 2018). Such enhancement can be mediated by microstructural changes, degradation of antinutritional factors such as phytic acid (Khan, Karnpanit, Nasar-Abbas, Huma, & Jayasena, 2018), as well as the synthesis or release of soluble ligands such as organic acids (Milman, 2020). However, there have also been reports of decreased endogenous Fe and Zn bioaccessibility in legumes after fermentation and germination (Luo, Xie, Jin, Wang, & He, 2014; Rekha & Vijayalakshmi, 2010). This may be in part attributed to the presence of the multimeric legume proteins, a large fraction of which is present in the hydrophobic β-sheet structure. These proteins may hinder the putative beneficial effects of bioprocessing on mineral bioaccessibility by being resistant to proteolysis and being aggregation-prone, particularly under higher concentrations of divalent cations (Ferreira, Freitas, & Teixeira, 2003). On the other hand, studies have reported that the enzymatic hydrolysis of legume proteins can generate soluble low molecular weight peptides that form soluble complexes with Fe and Zn (Evcan & Gulec, 2020). In these cases, Fe and Zn precipitation in the neutral environment of the small intestine would be prevented, favoring the elements absorption through both active uptake and passive diffusion across the intestinal epithelium (Sun et al., 2020).

The field pea (*Pisum sativum L*) constitutes the largest percentage of total current global pulse production at 36% (Lu, He, Zhang, & Bing, 2020). It possesses a valuable protein content between 23.1–30.9% (on a dry matter basis), and is comparable to soy proteins in having relatively high digestibility both *in vitro* (Le Roux et al., 2020) and *in vivo*

(Rutherfurd, Fanning, Miller, & Moughan, 2015). As it lacks the conglycinin fraction of soy protein that is known to be an inhibitor of iron absorption, hydrolysed pea proteins may form soluble complexes with Fe and/or Zn. Our preliminary *in vitro* study (Zhang, Stockmann, Ng, & Ajlouni, 2021) demonstrated that relative to the NaCl extract, the water extract of pea protein was able to effectively solubilize large quantities of Fe^{3+} during gastric, but not intestinal digestion, possibly due to residual phytate. Zn was found to be reduced in bioaccessibility in the presence of pea protein relative to the mineral salt control.

In the current study, we explored the effects of protein fractions obtained by enzymatic treatments or fermentation of pea protein on the bioaccessibility of fortified Fe and Zn salts through an *in vitro* digestion. Enzymatic hydrolysis using protease, phytase, and fermentation in the presence of lactic acid bacteria were used to generate hydrolysed fractions that could modulate the interactions of minerals with PPC constituents. *Lactobacillus plantarum* was chosen in this study due to existing evidence of its effectiveness in degrading phytate in legume matrices (De Pasquale et al., 2020), and high proteolytic activities towards some legume proteins (Rui et al., 2015). Both elements (Fe^{3+} and Zn^{2+}) were examined under an intermediate salt fortification concentration (15 mM), a dose that remains physiologically relevant at 83.8% and 75.4% of the daily Estimated Requirements for Australians for Fe and Zn, respectively.

5.2. Materials & Methods

5.1.1. General reagents and equipment

All chemicals used in the study were reagent grade and purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). The exceptions were the Pierce BCA Protein Assay Kit, pepsin and pancreatin, which were acquired from Thermo-Fisher Scientific (Scoresby, Victoria, Australia).

Lactobacillus plantarum [AB-Life (Lp1)] was purchased from Mylan Health (Millers Point, New South Wales, Australia). The other two subspecies of *Lactobacillus plantarum* [Bactoferm® Vege-Start 60 (Lp2) and Harvest LB-1 (Lp3)] were kindly provided by Chr. Hansen Pty. Ltd (Bayswater, Victoria, Australia). Protease M 'Amano' (protease peptidase from *Aspergillus oryzae, activity* 10,000 U/g) was kindly provided by Wilmar Bioethanol (North Sydney, New South Wales, Australia), and wheat phytase (6-phytase, activity 50 U/g as examined by manufacturer) was purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia).

Optical density was measured using a UV-Visible Multiskan™ GO from Thermo-Fisher Scientific (Scoresby, Victoria, Australia). All centrifugation were conducted on Allegra X-12R (Beckman Coulter, Mount Waverley, Victoria, Australia). All experiments were performed in triplicate.

5.1.2. Preparation of pea protein concentrate

A water-soluble pea protein concentrate (PPC) was prepared from dehulled field pea (*Pisum sativum L.*) in accordance with Makeri et al. (2017). In this method, PPC was extracted by suspending defatted pea flour in Milli-Q water (1:6, w/v, 2 hours), rested for 20 minutes and centrifuged at 3,500 × g for 20 min. The supernatant was decanted and dialysed against Milli-Q water using cellulose tubings (MW cut-off 10 kDa). Dialysis took place at room temperature for 4 hours with continuous stirring by a magnetic bar, and with the buffer changed every 2 hours before dialysing again overnight (12 hr) at 4°C. The dialysed solution was filtered using a cellulose membrane (10311897, Whatman, Thermo-Fisher Scientific, Australia) to reduce the insoluble solids formed during dialysis. The soluble dialysed fraction was pooled for bioprocessing, with total solids determined following the AOAC method using lyophilization (Emmons, Larmond, & Beckett, 2020).

5.1.3. Enzymatic hydrolysis of PPC

For the peptidase-only treatment, protease M was added to the dialysed and filtered concentrate at 400 U/g, or 4% w/w of solids equivalent as recommended by the manufacturer. For the phytase-only treatment, phytase was added to the dialysed and filtered concentrate at 0.8 U/g of the solids equivalent (Luo & Xie, 2012). Both phytase and peptidase were simultaneously added in the combined treatment. For each of the treatments, the reaction took place for 3 hours at 50 °C in an incubator (ZWYR-240, Labwit Scientific, Shanghai, China), with the pH adjusted to 5.0 every 45 minutes. The selected pH and temperature were within the optimal range reported in respective manufacturers' specifications. Enzymes were inactivated by bringing the pH to 10 using 1M NaOH before

isoelectric precipitation of the hydrolysed proteins at pH 4.5 and recovery by centrifugation at 3500 *x g* for 15 min. The hydrolysed fractions were frozen at −20°C and lyophilized.

5.1.4. Fermentation of PPC

The subspecies of *Lactobacillus plantarum* [(AB-Life (Lp1), Bactoferm® Vege-Start 60 (Lp2) and Harvest LB-1 (Lp3)] were enumerated in MRS broth at 30 °C for 24 h, and harvested by centrifugation at 2743 *x g* for 20 min (Rosa-Sibakov, Re, Karsma, Laitila, & Nordlund, 2018). The cell pellet was washed twice with distilled water and resuspended in sterile 0.1% peptone water to obtain a target cell density of 10^8 CFU mL^{-1}. Inoculation was carried out by adding 1 mL cell suspension to every 2.5 g solids equivalent of the dialysed and filtered peptide. Fermentation was performed for 24 h (30 °C), in sterilised glass bottles wrapped in aluminium foil. Proteins from the fermentation were isoelectrically precipitated by adjusting to pH 4.5, and recovered by centrifugation at 3500 *x g* for 15 min. The fermented fractions were also frozen at −20°C and lyophilized. The fermentation yielded three different fermented pea protein fractions, labelled Lp1, Lp2, and Lp3 representing the three subspecies of *Lactobacillus plantarum,* respectively.

5.1.5. Mineral-binding and simulated digestion

5.1.5.1. Preparation of element-containing legume extract solutions

Freeze-dried concentrates of each PPC were dissolved in 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer stirring overnight at 4°C, at pH 7 for Fe, and 8 for Zn (Eckert et al., 2014). The final concentration was 1% (w/v). The element solutions for *in vitro* digestion ($FeCl_3$ and $ZnCl_2$) were prepared at 15 mM, with 5 mL was

used in each run. These salts were chosen due to their high solubility in water. The element solutions were then stirred for 20 min with either the 1% protein concentrate (w/v) or control solutions of 0.1 M HEPES (10 mL) without proteins. The final concentrations of 4.19 mg Fe or 4.9 mg Zn with or without 100 mg of PPC, as 15 mL in total were subjected to the *in vitro* digestions.

5.1.5.2. Simulated oral digestion

A procedure adapted from the INFOGEST model (Egger et al., 2016), based on a modified version of simulated salivary fluid was employed in the current study. The simulated salivary fluid contained 0.15% NaCl, 0.02% $CaCl_2 \cdot 2H_2O$, 0.3% KH_2PO_4, 0.15% mucin and 0.01% bovine serum albumin, which was formulated in our previous study based on biochemistry values from human stimulated/non-stimulated saliva (Zhang et al., 2021). The salivary fluid used was different from that of the INFOGEST model as the new formulation accounts for the various ionic salts present in the salivary fluids that may affect element solubility, whereas the original formulation contained only $CaCl_2$. After adjusting to pH 6.8, the solution was stirred and preheated to 37 °C as outlined in the INFOGEST model. However, the solutions of element-containing legume extracts or control (15 mL HEPES buffer) were mixed with 5 mL of salivary fluid in the current model. This is different from the 1:1 (sample:saliva) proportion used in INFOGEST to avoid aggregation of the additional mineral salts. The mixture was shaken using an incubator shaker (ZWYR-240, Labwit Scientific, Shanghai, China) at 37 °C for 3 min to mimic agitation in the mouth.

5.1.5.3. Simulated gastric and small intestinal digestion

We have briefly modified a two-step digestion system from our previous work (Zhang et al., 2021). Following oral digestion, 5 mL of simulated gastric fluid containing 34 mM NaCl, HCl (pH 1.2) and pepsin (6.4 mg/mL) was mixed with each sample, and incubated in the shaking incubator for 2 h at 37 °C at 2 *x g*. Digestion was ceased by adjusting sample pH to 6.8 with 1 M NaOH. Digestion triplicates were continued onto intestinal digestion immediately.

The intestinal electrolyte fluid constituted 5 mM PBS, 0.4 M NaCl and 15 mM $CaCl_2$ and was adjusted to pH 6.8 (± 0.05). Each gastric digested replicate was mixed with 7 mL of the prepared simulated intestinal fluid. The electrolyte fluid and gastric digesta mixtures were then gently stirred with added pancreatin (10 mg/mL of digestive fluid) and bile (0.5 or 30 mg/mL of digestive fluid) as respective proxies of the concentrations secreted by the gall bladder during fasting (absence of food) and fed (presence of food) conditions. All samples were placed in a shaking incubator for 2 h (fasted) and 3 h (fed) at 37°C and 2 *x g*, with digestion was ceased by placement in an ice bath. The samples were then centrifuged for 10 minutes at 2465 *x g*, and the soluble supernatant fractions were subjected to analyses for soluble protein, degree of protein hydrolysis and soluble mineral content.

The positive (mineral salts only) and negative (protein with no mineral salts) controls were also subjected to the gastrointestinal model for comparison amongst samples (proteins + mineral salts). The negative control was subtracted from samples during calculations of soluble minerals.

5.1.6. Protein analyses

5.1.6.1. Total soluble protein

The soluble protein contents of the reconstituted fractions from the small intestinal digestion were determined by the bicinchoninic acid method (BCA), following a commercial kit with bovine serum albumin as the standard (Pierce BCA Protein Assay Kit, Thermo-Fisher Scientific).

5.1.6.2. Free α-amino groups

The number of free α-amino groups from proteins at the end of intestinal digestion was determined with an adapted protocol using the *o*-phthaldialdehyde (OPA) reagent (Nielsen et al., 2001; Zhang et al., 2019). The composition of the OPA reagent was 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. To each 10 µL aliquot of protein sample, 240 µL OPA reagent was added and incubated for 2 minutes at room temperature before absorbance measurement at 340 nm. The meqv. of serine released per gram of protein (mg Serine-NH_2) was calculated as follows (Zhang et al., 2019):

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

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 above.

6.2.6.3. SDS-PAGE

The molecular weight distribution of the native and bioprocessed PPC was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli (1970). For sample preparation of the non-reduced native proteins, PPC and its hydrolysates (10% w/v, resuspended in HEPES buffer) were diluted 1:1 in volume with sample buffer (containing 65.8 mM Tris–HCl, 2.1% SDS (w/v), 26.3% glycerol (v/v), 0.01% bromophenol blue, pH 6.8), and heated for 5 min in a heating block at 85°C (DBH2000D, Ratek Instruments, Boronia, Australia). For the reduced sample, a factor of 1:0.95 (v/v) was used for dilution with sample buffer, with 2-mercaptoethanol added for a total sample to buffer ratio of 1:1 (v/v). Following these steps of protein denaturation with SDS, aliquots (15 µl) were transferred into the wells of precast 4%–20% polyacrylamide gels (Mini-PROTEAN TGX Precast gels, Bio-Rad Laboratories). The samples were separated for 40 min at 200 V (60 mA, 100 W) (Amersham Biosciences, Buckinghamshire, UK) at room temperature in a vertical electrophoresis cell (Bio-Rad Laboratories). Precision Plus Protein Unstained Standard with molecular weight of 10–250 kDa (Bio-Rad Laboratories) run alongside as size markers. The values were obtained by graphic interpolation.

The two active bioprocessed fractions shown to have enhanced Fe and Zn bioaccessibility were pooled and concentrated *via* ultrafiltration centrifugal columns (10 kDa, Centriprep® centrifugal filter unit, Merck-Millipore, Darmstadt, Germany). The > 10 kDa fraction (retentate) for the two samples were subjected to SDS-PAGE to examine the MW profile after simulated digestion.

5.1.7. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

5.1.7.1. Acid digestion

Acid digestion was conducted based on our previous work (Zhang, Panozzo, Hall, & Ajlouni, 2018). For each sample, a 2 mL aliquot from the soluble supernatant by the end of intestinal digestion 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. The samples were then vortexed and pre-digested for 12 hours at room temperature, followed by heating on an aluminium digestion block with Proportional-Integral-Derivative control (Custom made block, The University of Melbourne, Australia). The operational parameters for the block was 30 min at 80 °C, and then increased to 125 °C for 120 min at 5 °C intervals. Following digestion, the samples were diluted to an acid content of < 5% using MilliQ- grade water (30 mL total) in alignment with the 5% HNO_3 calibration standard matrix used.

5.1.7.2. ICP-OES Specifications

ICP analyses were performed using a radial view Optima 8300 DV ICP-OES (Perkin Elmer, Glen Waverley, Australia). Samples were injected using an automated sampler (S10, Perkin Elmer, 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.

5.1.8. 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. One-way ANOVA was performed for Fe and Zn for each digestion phase (fasted or fed) through the 'General Linear Model' function, using Dunnett's post-hoc with PPC as the control. One-way ANOVA with Fisher's LSD was also utilized to assess for differences in soluble protein or free amino groups between the fractions.

5.3. Results

5.3.1. Elemental bioaccessibility

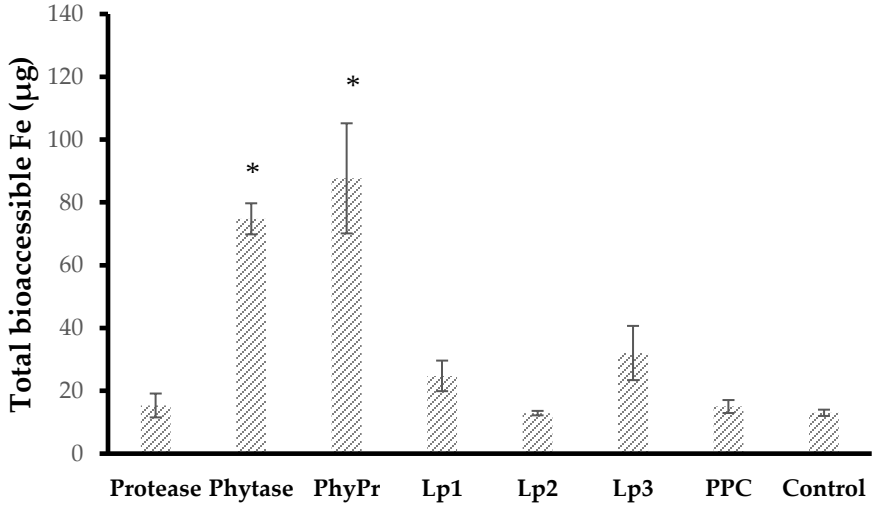
5.3.1.1. Iron

The analyses of soluble, hence bioaccessible Fe were performed on samples following sequential digestion of oral, gastric and the small intestines. Results in **Figure 10** showed that less than 5% of the Fe loaded (4190 µg) was soluble in the control, during both fasted and fed stages of the small intestinal digestion.

Fe^{3+} solubility in the presence of PPC was only slightly higher than the control with 15 ± 2 and 13 ± 1 µg during fasted and fed digestion stages, respectively. Treatment of PPC with protease only showed a small enhancement of Fe bioaccessibility in fed digestion, and with no significant effect in fasted digestion, compared to untreated PPC (**Figure 10**).

Phytase (Phy) treatment of the PPC had a significant ($P < 0.05$) enhancement effect on Fe bioaccessibility. The amount of soluble Fe reached 75 ± 5 and 146 ± 17 µg of Fe in the fasted and fed digestion stages, representing 5- and 12-fold enhancement over PPC, respectively. Bioaccessibility was further enhanced by combining phytase and protease treatments (PhyPr) of PPC, increasing Fe solubility to 88 ± 18 µg in fasted and 184 ± 25 µg in fed digestions. These are equivalent to 2.1% and 4.4% of the total fortified Fe (4190 µg) and representing 6- and 15- fold increases over the untreated PPC, respectively.

a)



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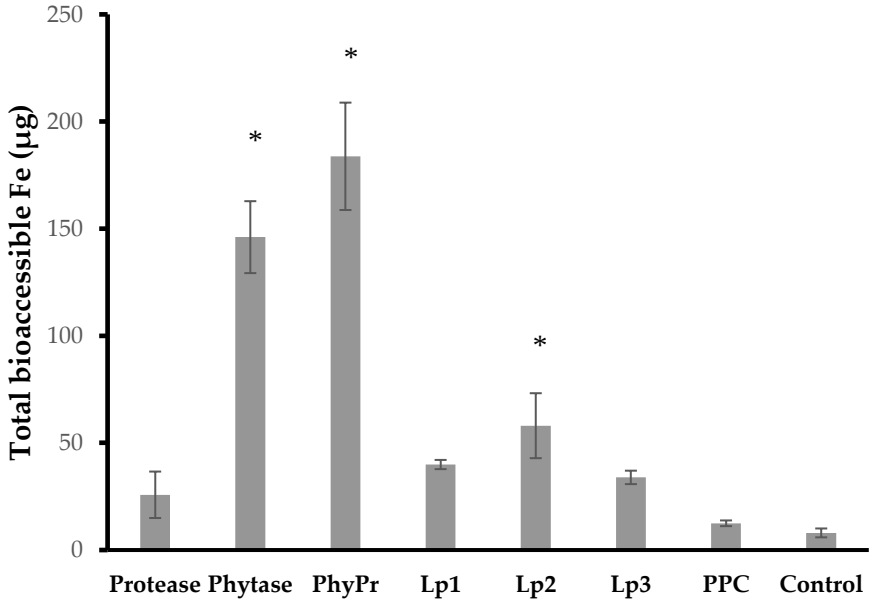
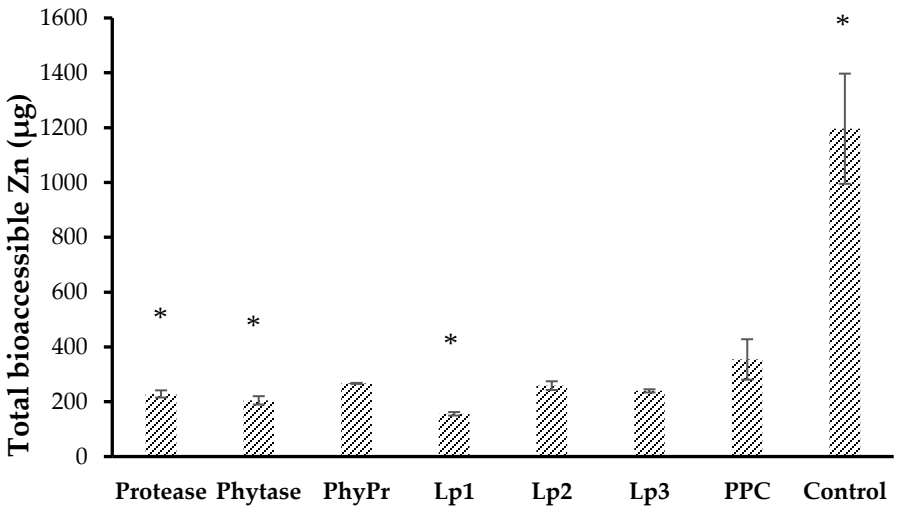


Figure 10. Total bioaccessible Fe after a) fasted and b) fed stages of digestion. Fe loading was 4190 µg and bioaccessibility, accessed as soluble Fe in the supernatant of centrifuged samples, expressed as means ± SD (n = 3). PPC, pea protein concentrate. Treatments were: Protease treated PPC (Pr), phytase treated PPC (Phy), combined protease + phytase treated PPC (PhyPr), Lp = *Lactobacillus plantarum* treated PPC (sub-species Lp1, Lp2,and Lp3). Control ($FeCl_3$ only). An asterisk (*) indicates significant difference ($P < 0.05$) in comparison with pea protein concentrate (PPC).

a)



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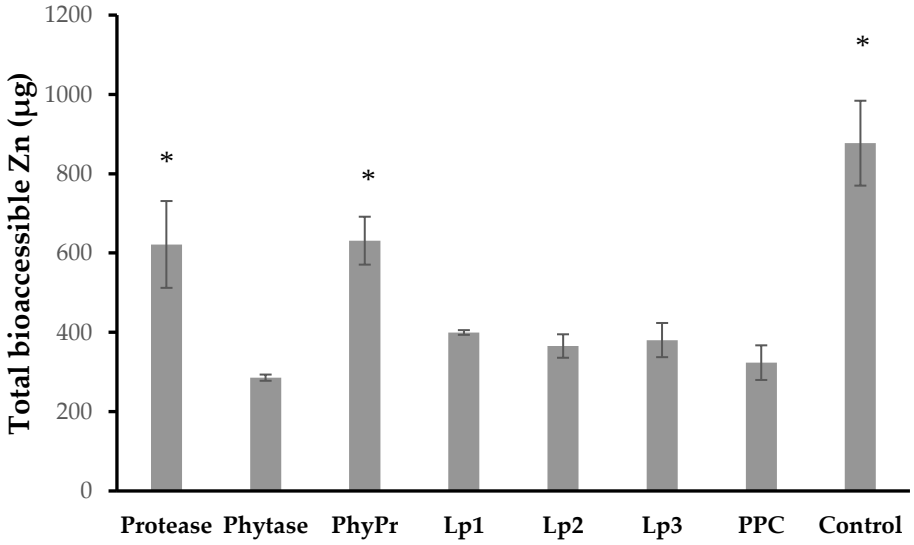
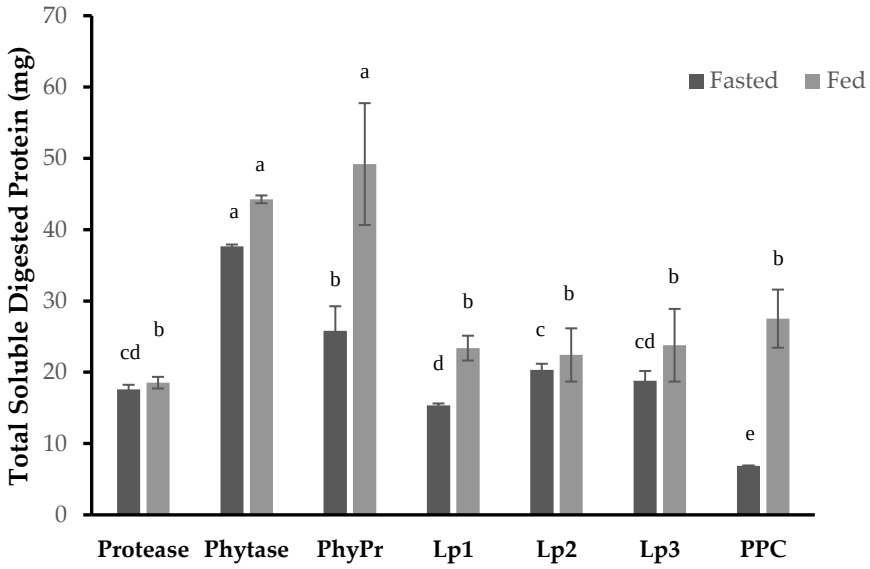


Figure 11. Total bioaccessible Zn after a) fasted and b) fed stages of digestion. Zn loading was 4900 µg and bioaccessibility, accessed as soluble Zn in the supernatant of centrifuged samples, expressed as means ± SD (n = 3). PPC, pea protein concentrate. Treatments were: Protease treated PPC (Pr), phytase treated PPC (Phy), combined protease + phytase treated PPC (PhyPr), Lp = *Lactobacillus plantarum* treated PPC (sub-species Lp1, Lp2, Lp3). Control ($ZnCl_2$ only). An asterisk (*) indicates significant difference ($P < 0.05$) in comparison with pea protein concentrate (PPC).

a)



b)

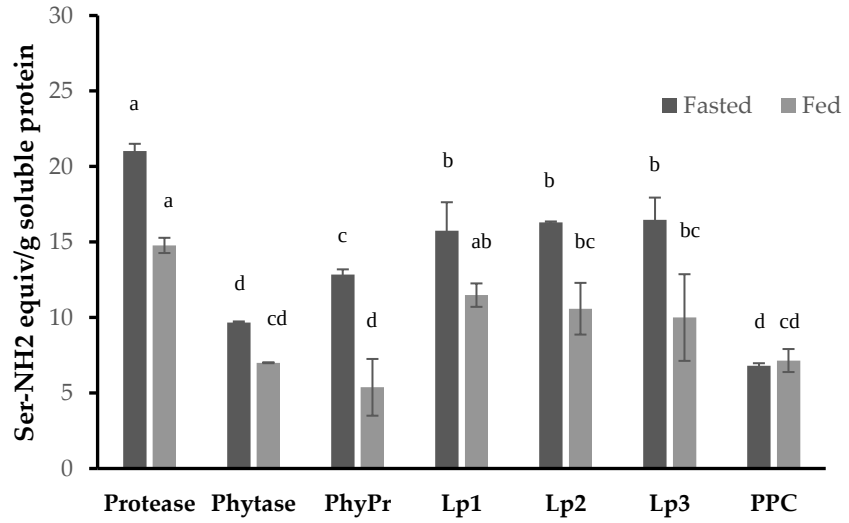


Figure 12. Total soluble protein (a) and free α-amino groups per gram soluble protein (b) present in the soluble supernatant after fasted and fed stages of digestion, for Fe fortified samples. Values are from the 100 mg PPC powder administered and expressed as means ± SD (n = 3). Column with different superscript letter within each state of digestion indicates significant differences ($P < 0.05$). Treatment abbreviations were: PhyPr = combined protease + phytase, Lp = *Lactobacillus plantarum.*

Interestingly, *Lactobacillus plantarum* 2 (subspecies Bactoferm® Vege-Start 60, Lp2) fermented PPC also enhanced Fe bioaccessibility, with the amount of soluble Fe being 58 ± 15 µg. This is equivalent to a 4.8-fold increase over untreated PPC (**Fig 10-b**), albeit was much less effective than phytase or phytase-protease treat PPC. However, this enhancement effect was not seen with the other subspecies, AB-Life (Lp1) or Harvest LB-1 (Lp3) (40 ± 2 and 34 ± 3 µg, respectively).

5.3.1.2. Zinc

The solubility of Zn, hence bioaccessibility, was much higher than Fe fortified at the same level during both Fasted and fed intestinal digestions. During fasted and fed digestion stages, 24.4% (1196 ± 201 µg out of 4900 µg of fortified Zn) and 17.9% (877 ± 107 µg out of 4900 µg) were soluble, respectively.

Conversely to that observed with Fe, PPC and all treated PPC led to decreased Zn bioaccessibility compared to Zn control (**Figures 11-a and 11-b**). When PPC was included in fasted and fed digestion, 7.2% (354 ± 74 µg) and 6.6% (324 ± 44 µg) were soluble, respectively.

However, divergent effects from bioprocessing of PPC were observed between the two stages of intestinal digestion. During fasted digestion, bioprocessing of PPC had no significant ($P > 0.05$) promotive effect relative to PPC without bioprocessing (**Figure 11-a**). Both enzymatic treatments, Pro and Phy, showed significantly ($P < 0.05$) lower Zn bioaccessibility during the fasted state of digestion compared with the native PPC (**Fig 11-**

a). The Zn bioaccessibility under these conditions were Pro at 4.6% (228 ± 13 µg) and Phy at 4.2% (205 ± 15 µg), compared to untreated PPC at 7.2% (354 ± 74) µg.

PPC treated with *L. plantarum 1* (mixed subspecies AB-Life, Lp1) also exhibited significantly lower Zn bioaccessibility (3.2%, 156 ± 6 µg) in the fasted state compared to untreated PPC (**Fig 11-a**). Meanwhile, no significant differences in Zn bioaccessibility were found between PhyPro-treated PPC and untreated PPC, or from any of the other fermented pea fractions (Lp2 and Lp3) during fasted digestion. During fed digestion, the presence of PPC led to lower Zn bioaccessibility relative to the salt control (Fig 2b, PPC: 323 ± 44 µg, Control: 877 ± 107 µg). Pro (621 ± 109 µg) and PhyPr (631 ± 60 µg) treatments were able to promote Zn bioaccessibility when compared to native PPC (**Fig 11-b**).

5.3.2. Protein analyses

5.3.2.1. Soluble Protein and free α-amino groups

5.3.2.1.1. Iron

The quantities of soluble proteins in the centrifuged supernatant following intestinal digestion in the presence of Fe salt are illustrated in **Figure 12**. Out of the 100 mg of PPC powder administered, the largest amounts of soluble proteins were detected in the PhyPro treated PPC (49.2 ± 8.5 mg) followed by Phy treated PPC (44.2 ± 0.5 mg) during the fed state of digestion (**Fig. 12a**). However, an opposite trend was observed under the fasted state of digestion with significantly larger amounts of soluble protein in the Phy treated PPC (37.6 ± 0.27 mg) than in the PhyPro treated PPC (25.8 ± 3.4 mg). The same data also revealed that under both states of digestions the quantities of total soluble proteins in both

Phy and PhyPro treatments were significantly ($P < 0.05$) larger than all other treatments (Pro, Lp1, Lp2, Lp3 and PPC) from the 100 mg PPC administered. It should be noted here that these two enzyme hydrolysates (Phy and PhyPro) produced greater amounts of Fe bioaccessibility (**Fig 10**).

The number of free α-amino groups per gram soluble protein reported as mg serine-NH_2 equivalents ranged from 6.8 to 21.03 mg in PPC and Pro treated PPC, respectively, during fasted digestion, and 5.38 in PhyPro treated PPC to 14.8 mg in Pro treated PPC during fed digestion in the presence of fortified Fe (**Figure 12-b**). It was observed that during fasted digestion all bioprocessing treatments, except PPC and Phy treated PPC, significantly increased the number of free α-amino groups per gram of soluble protein (**Fig. 12-b**). During fed digestion, only Pro treated and Lp1 fermented PPC showed significantly ($P < 0.05$) greater number of free α-amino groups per gram soluble protein (Pro: 14.8 ± 0.5 mg, Lp1: 11.5 ± 0.78 mg) when compared with untreated PPC (7.15 ± 0.76 mg).

5.3.2.1.2. Zinc

In the presence of fortified Zn, the total solubilized proteins from the 100 mg of untreated PPC powder ranged from 7.6 mg to 35.5 mg (Phy treated PPC) during fasted stage, and from 20.3 mg to 39.7 mg (PhyPro treated PPC) during fed stage digestion (**Fig 13-a**). The same data also demonstrated that there was a significant ($P < 0.05$) increase in total soluble protein in all bioprocessed samples except the unprocessed PPC treatment following fasted intestinal digestion. However, the significant increment in the total soluble protein under the fed state conditions were detected only in Pro, Phy and PhyPro treatments. The reported

amounts of soluble proteins in these treatments were Pro: 26.8 ± 4.72 mg, Phy: 34 ± 0.15 mg and PhyPro: 39.7 ± 1.57 mg. Out of the 100 mg of administered PPC, there was no significant differences ($P > 0.05$) in soluble protein contents between the PPC and its fermented counterpart samples after digestion.

All bioprocessing treatments led to a significantly ($P < 0.05$) higher number of free α-amino groups per gram soluble protein in comparison with the untreated PPC. For example, the amount of free α-amino groups reported as mg serine-NH_2 equivalents in fed state ranged from 10.5 mg (Lp3) to 13.3 mg (Lp2) vs 0.65 mg in untreated PPC. The fasted state of digestion showed a greater range of free α-amino groups per g of soluble protein relative to the fed counterparts (**Fig. 13-b**). During fasted digestion, the mg serine-NH_2 equivalents ranged from 5.4 to 23.3 mg in untreated and Pro treated PPC, respectively. The fed state of digestion ranged from 0.65 to 13.3 mg in untreated and Lp2 treated PPC, respectively.

5.3.2.2. SDS-PAGE

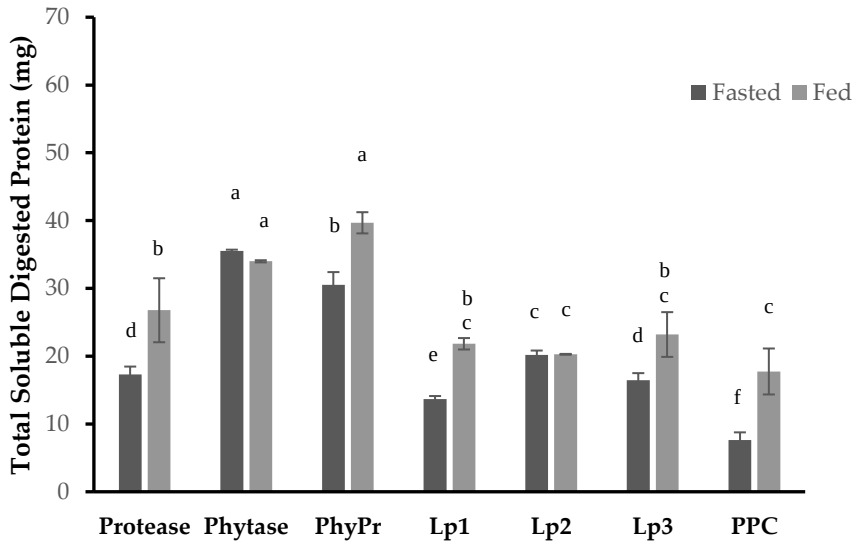
The characterisation of unprocessed and bioprocessed PPC was performed using SDS-PAGE. Results in **Fig 14** showed that the untreated PPC profile was identified by the presence of proteins in the range of 18 – 85 kDa, indicating the presence of both albumins and globulins (**Fig. 14-a**). Hydrolysis by Pro resulted in breakdown of the main proteins at 37 and above 50 kDa, and no apparent differences can be observed between non-reducing or reducing SDS-PAGE. Albumins (14 kDa), lectin (49 kDa), a mix of legumin (23 – 34 kDa, acidic/basic polypeptides) and vicilin (β-γ site – 19 and 33 kDa) protein bands were observed in Phy and Pro treated but not in PhyPro teated PPC. PPC treated with PhyPro

resulted in weak bands indicating extensive hydrolysis into smaller peptides/amino acids, including around 14 kDa (albumins), and 22-23 kDa, which are likely to be legumin basic polypeptides.

The profiles of PPC fermented with *L. plantarum* spp. revealed similar end products among the three sub-species (**Fig. 14-b**). No difference between the end products of fermentation were observed among Lp1, Lp2, and Lp3. Relative to the enzyme-treated PPC, Untreated PPC showed similar bands at a varying range of molecular weights between 10 – 138 kDa. However, some bands could be seen at ~140 kDa that are not present in the bioprocessed PPC. Some other low MW peptides (~12.5, 13.5 and 16 or 19 kDa), such as vicilin were also present. Peptides cleaved at both α-β and β-γ were present around the range of 10 kDa in the reduced profile of fermented PPCs, which can be identified as albumins (~10-18 kDa). Native lectin also appears to still be intact in this sample (~49 kDa).

As Phy and Pro treatments have demonstrated enhanced Fe and Zn bioaccessibility under fasted and fed conditions, respectively (**Figs. 10 and 11**), their samples after digestion in the model system were also characterised by SDS-PAGE after ultrafiltration with a 10 kDa membrane (**Figs. 15-a and 15-b**). Blank samples of Phy and Pro without salt addition were also run for comparison. The profile of the Phy + Fe showed bands at 18, 20, 37 and 50 kDa in the presence of the mineral salt vs. none without (**Figure 15-a**), indicating some peptide aggregation in the presence of the Fe salt. For Pro + Zn, there appears no significant differences ($P > 0.05$) in the MW profile without the addition of salt under both reduced and non-reduced conditions (**Figure 15-b**).

a)



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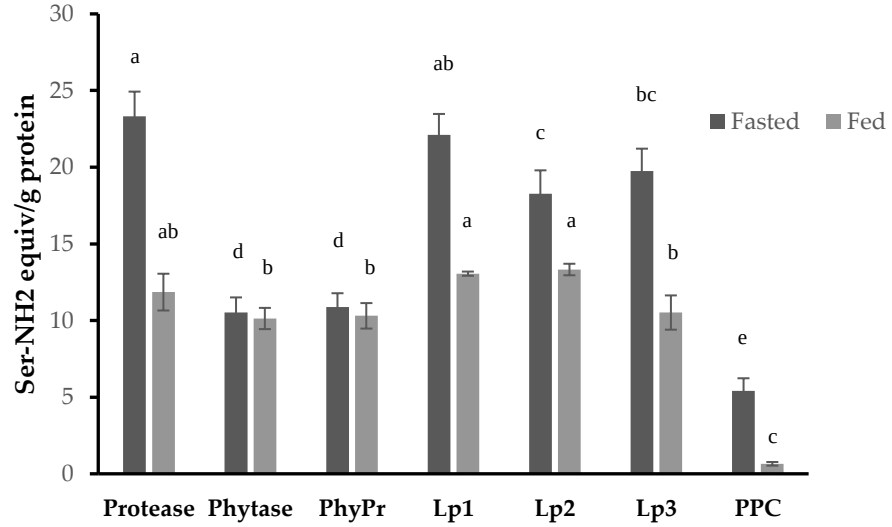
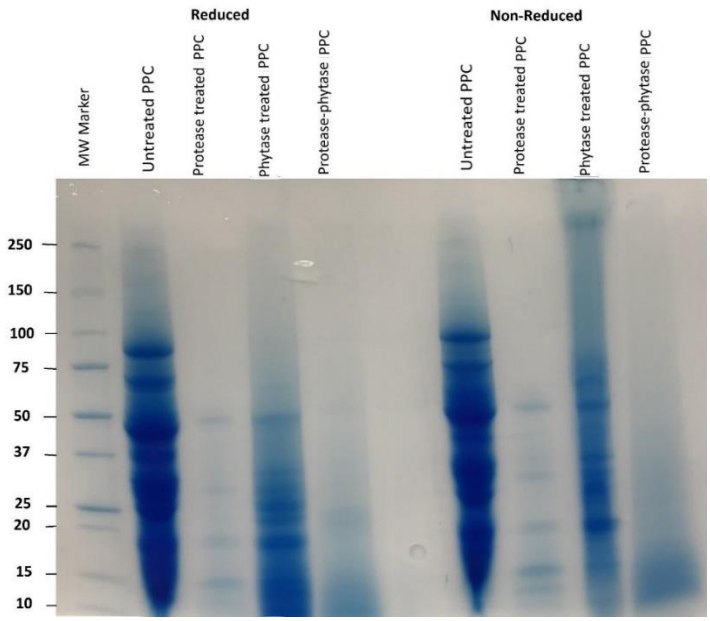


Figure 13. Total soluble protein (a) and free α-amino groups per gram soluble protein (b) present in the soluble supernatant after fasted and fed stages of digestion, for Zn fortified samples. Values are from the 100 mg powder administered and expressed as means ± SD (n = 3). Column with different superscript letter within each state of digestion indicates significant differences ($P < 0.05$). Treatment abbreviations were: PhyPr = combined protease + phytase, Lp = *Lactobacillus plantarum.*

a)



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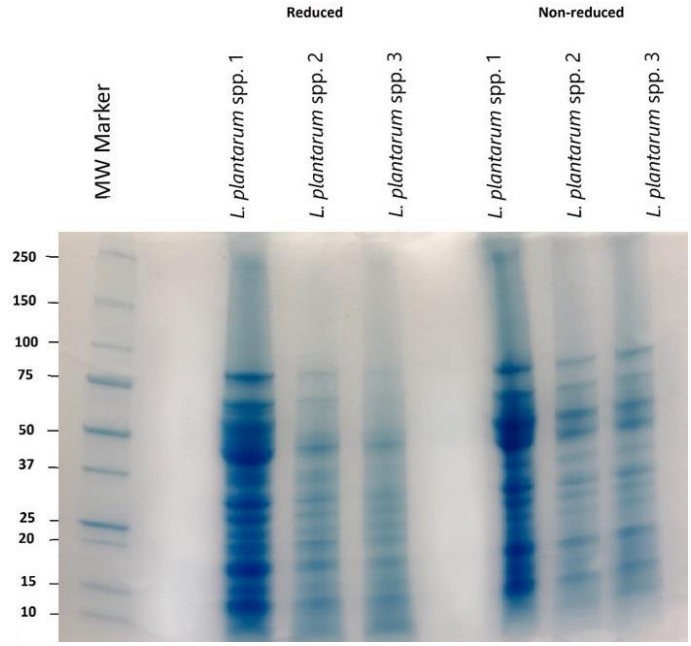
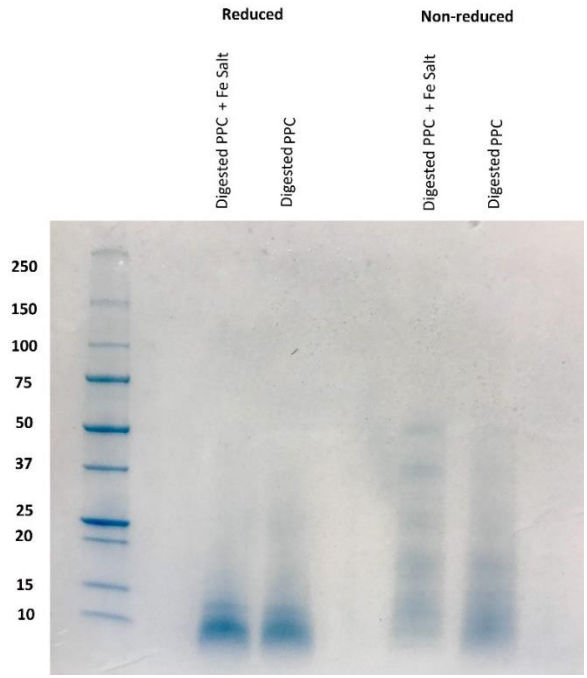


Figure 14. Reduced and non-reduced SDS-page profiles of a) non-digested pea albumin (PPC) in its native form, and treated with phytase, protease, and combined treatments, b) profile of non-digested pea albumin treated with the three *L. plantarum* strains. Lane 1 is the MW marker in kDa.

a)



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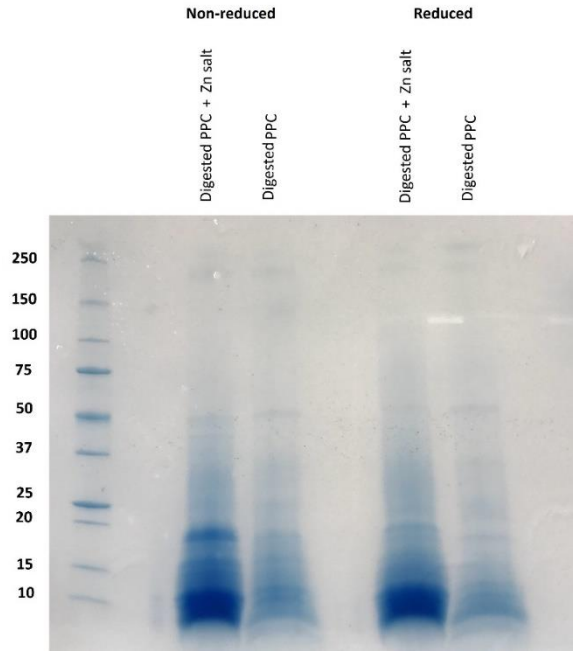

Figure 15. SDS-page profile of a) in vitro digested (fasted condition) PPC treated with phytase, with or without added Fe salt. b) profile of *in vitro* digested (fed condition) pea albumins treated with protease, with or without added Zn salt. Lane 1 is the MW marker in kDa.

5.4. Discussion

The bioaccessibility of non-heme Fe and Zn which are required for their absorption are limited, due to a series of physicochemical and physiological events that occur during the digestion process where food proteins can behave as a double-edged sword. Whilst many soluble di-and trivalent ion-binding peptides have been identified in food, the co-ingestion of some proteins such as casein and soy can inhibit mineral bioaccessibility. Pea proteins possess advantageous properties in relation to their amino acid profile (containing Fe- and Zn- binding residues such as His), with relatively high solubility and cost-production efficiency. However, crude legume seed storage proteins are also prone to forming aggregates with cations (Poulson et al., 2020; Santos, Alves, Bento, & Ferreira, 2012), which can lead to insoluble complexes where protein digestibility is impeded by the presence of phytic acid (bound to the protein) and proteolytic enzyme inhibitors. To our knowledge, this current study is the first to examine the effects of various bioprocessing approaches on pea protein concentrate (PPC) as a carrier targeting improving solubility, hence bioaccessibility, of relatively high concentrations of fortified Fe^{3+} and Zn during digestion.

Phytases can indirectly enhance proteolysis through the hydrolysis of phytate, which may increase the generation of soluble peptides that can bind Fe or Zn increasing their solubility. Our results demonstrated that treatments of PPC with phytase (Phy) and a combination of phytase and protease (PhyPro) are most effective at enhancing Fe^{3+} solubility, hence bioaccessibility. These results were in agreement with the findings from a rodent study by Urbano et al. (2007), which showed that phytase treatments of pea flour proteins could

enhance endogenous Fe bioavailability. Our data suggest that this effect occurs in conjunction with higher levels of soluble protein when phytase treated PPC is digested, and was observed during both fasted and fed digestion stages (**Fig. 10**). As previously mentioned, it is likely that phytase can hydrolyse phytate and thereby promote the release of peptides that can form soluble complexes with Fe^{3+} during digestion, which was not evident by proteolysis from added or digestive proteases only. It is understood that under the conditions of the small intestine, cations such as Fe^{3+} can mediate protein-phytate complexes at pH levels above the isoelectric point of proteins. Additionally, such cations can bind with some peptide fragments generated during digestion. Consequently, it can be postulated that by treating legume proteins with phytase prior to ingestion, mineral binding can be reduced by partial dephosphorylation of the hexaphosphates, which have less pronounced binding strength and/or capacity for cations (Gupta et al., 2015; Zhang, Stockmann, Ng, & Ajlouni, 2020). Cations can act as bridging ions in aggregate formation and decreases both mineral and protein solubility. In supporting our interpretation, the SDS-PAGE profile of Phy treated PPC (**Fig. 15-a**) indicated that despite some Fe-mediated peptide aggregation observed involving disulphide bonds, the pea proteins were extensively hydrolysed to peptides < 15 kDa following *in vitro* digestion.

It is generally believed that bacterial fermentation of plant-derived foods containing phytic acid can promote iron bioaccessibility during digestion, in part due to bacterial phytase activity (Sulibursk a & Krejpcio, 2014). We found that the fermentation of PPC using some lactic acid bacteria generally did not lead to any significant effects ($P > 0.05$) on Fe salt solubility during fasted digestion. Although one of the lactic acid bacteria sub-species did

show solubility enhancement effects under fed digestion conditions, it was still lower compared to enhancement effects produced by phytase-containing enzymes. Our results are in agreement with the mixed findings from *L. plantarum* fermented legumes on endogenous Fe reported in the literature. For example, Rekha and Vijayalakshmi (2010) reported a reduction in the Fe bioaccessibility when soy protein was fermented with *L. plantarum* and yeast *S. boulardii*, whilst Sulibursk a, Krejpcio, Lampart-Szczapa, and Wójciak (2009) showed an enhancement with *L. plantarum, L. brevis, and Leukonostoc mesenteroides* fermentation of lupin protein.

Since the current study investigated different *L. plantarum* subspecies under the same conditions, it is likely that they produced varying effects on the PPC matrix that led to divergences in Fe bioaccessibility. Our findings also suggest that this difference may not be directly linked to the quantity of soluble proteins, or the MW distribution profile between the range of 10 – 250 kDa. This could be attributed to the fact that most food-derived soluble Fe-binding peptides are < 1.5 kDa (Wu, Yang, Sun, Bao, & Lin, 2020). Consequently, extensive proteolysis of the peptides must occur during the fed digestion to ensure better Fe binding. Additionally, there could also be variance in the intrinsic enzymatic properties of the *L. plantarum*, specifically that of phytase activity (Zamudio, Gonzalez, & Medina, 2001) leading to variable degrees of phytate hydrolysis between subspecies.

Similar to the reported observations with Fe bioaccessibility, enzymatic hydrolysis and fermentation of PPC led to intriguing differences in Zn bioaccessibility. Whilst two of the

enzymatic treatments (Phy and Pro) led to Zn bioaccessibility promotive effects on during fed, but not fasted digestion, fermentation did not enhance Zn bioaccessibility (**Fig. 11**). Whilst there has not been any study about the impact of enzymatic hydrolysis on Zn bioaccessibility in pea protein, our results corresponded with previous investigations that have employed soy protein. Using proteases to hydrolyze soy, Devaraju, Thatte, Prakash, and Lakshmi (2016) also showed a promotive effect on Zn bioaccessibility. Furthermore, enhancements of Zn bioaccessibility using phytase treatment of soy was reported by Theodoropoulos, Turatti, Greiner, Macedo, and Pallone (2018). These two studies have utilized similar quantities of bile to the fed state in their digestion model. This indicates the effect of bile with digestion time in supporting Zn bioaccessibility, which may be achieved through the release of lower MW Zn-binding peptides during fed digestion that were not bioaccessible during fasted digestion. Bile in particular also possesses surfactant effects that confer more stability and inhibit some intermolecular aggregation forming peptides (Corte-Real & Bohn, 2018). Results from the SDS-PAGE revealed that the protease-treated PPC had a similar digestion profile with and without the Zn salt, indicating little aggregation of the Zn-peptide complexes after fed digestion (**Fig. 15-b**).

The fermentation of PPC was found to have a neutral or inhibitory effect on Zn bioaccessibility, a finding that simultaneously supports and contradicts previous studies. Several authors have reported enhancements from lactic acid bacterial fermentation on *in vitro* endogenous Zn bioaccessibility in legume protein matrices (Agte, Gokhale, & Chiplonkar, 1997; Rekha & Vijayalakshmi, 2010). However, a reduction in Zn bioaccessibility as a result of legume fermentation can also occur, but this depended on the

type of legume used (Hemalatha, Platel, & Srinivasan, 2007). These studies have all utilized different cultures, legumes and digestion protocols involving none or varying levels of bile. These parameters may have contributed to the discrepancies observed in Zn bioaccessibility, as they influence factors such as the amount and type of peptides, and anti-nutrients released during fermentation. For example, *L. plantarum* fermentation of PPC has been shown to increase total tannin and phenolic content due to matrix liberation (Çabuk et al., 2018), which may interfere with the solubility of Zn-peptide complexes. It was noted from our study that whilst the total solubilized proteins in the presence of Zn was lower than that of Fe, the degree of hydrolyses per g of soluble protein was comparable if not higher than that of Fe. This shows that whilst the number of binding sites was increased in samples digested in the presence of Zn, some of the Zn-peptide complexes may not have been soluble by the end of intestinal digestion, leading to no or inhibitory effects on Zn bioaccessibility.

For the first time, we report the effects of enzymatic hydrolysis and fermentation using *L. plantarum* on PPC as a carrier for fortified Fe and Zn. Enzymatic hydrolysis involving phytase enhanced Fe^{3+} bioaccessibility regardless of digestion conditions, whilst fermentation was generally ineffective, though one strain produced promotive effects during fed-digestion phase. Enzymatic or fermentation treatments led to no effect, or reduced bioaccessibility of the Zn salt during fasted condition. However, treatments containing protease were effective at enhancing Zn bioaccessibility during fed stage digestion. More research is needed to confirm the mechanisms underlying the effects of bioprocessing approaches on Fe and Zn bioaccessibility in legume protein-rich matrices.

The divergence observed between the fasted and fed conditions underlines the role of different simulated digestion parameters in *in vitro* studies examining elemental bioaccessibility. This can lead to mixed results in addition to differences in the element concentration and bioprocessing conditions utilized. Whilst a standardized *in vitro* simulated digestion protocol exists in the literature (Egger et al., 2016), the INFOGEST protocol may require adaption when investigating elemental bioaccessibility. This proposition is based on the presence of additional endogenous mineral salts in digestive fluids and varying bile concentrations may further contribute to ionic strength and aggregation of mineral salts. These are properties that govern essential elemental bioaccessibility in these simulation systems, and possibly *in situ* during digestion.

Chapter 6. Characterisation of Fe(III)-binding peptides in pea protein hydrolysates

Chapter 5 identified that enzymatic hydrolysis was the most effective bioprocessing method at enhancing Fe(III) bioaccessibility, where the effects of bioprocessing on Zn bioaccessibility were inconsistent. As such, this chapter aims to isolate and identify the peptides within the pea protein hydrolysates potentially responsible for solubilising iron. Based on the SDS-PAGE profiles generated after *in vitro* digestion in the previous chapter, it was confirmed that the putative soluble Fe- and Zn-binding peptides linked to enhanced elemental bioaccessibility were of MW < 10 kDa. These peptides were largely produced following both phytase and protease hydrolysis.

This chapter firstly examined the iron-binding capacity of the impurified pea enzymatic hydrolysate using the two aforementioned enzymes. The iron-binding peptides were then separated with an Fe(III)-charged immobilized metal affinity column. The resultant fraction with and without complexation with additional Fe (as $FeCl_3$) were characterised through FTIR analysis. Finally, bottom-up LC-MS/MS proteomics techniques were used to identify the isolated peptides. Further bioinformatic analyses were performed to obtain insight towards the physicochemical properties of these peptides.

The chapter contains the third research study as published in *Food Chemistry*. The version included has been peer-reviewed without editorial copyediting, typesetting and proofreading. The full reference can be found in the preface of the thesis.

Characterization of Fe(III)-binding peptides from pea protein hydrolysates targeting enhanced iron bioavailability

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ABSTRACT:

This investigation aimed to characterize enzymatically-derived pea peptides that act as solubilizing agents to enhance Fe(III) bioaccessibility. The pea hydrolysates were found to have an iron-binding capacity of 5.3 mg/g lyophilized powder. The Fe(III)-binding peptides were separated by immobilized metal affinity chromatography (IMAC), and then sequenced using tandem MS following an in-solution tryptic digestion. Results revealed that the Fe(III)-binding fraction was rich in Glu, Asn, Lys and Leu, and the peptides primarily belonged to the Vicilin family. After screening based on the peptides' relative abundance and physicochemical properties, 15 novel peptides below 1.5 kDa were identified as potential candidates for enhancement of iron bioavailability. Fourier

Transform infrared spectroscopy (FTIR) of the hydrolysate-iron complex suggested that the principal sites of peptide binding corresponded primarily to the carboxylate groups, with amine I and II groups also evident.

6.1. Introduction

Iron is an essential trace element required for numerous metabolic activities and processes involved in body function, including cellular respiration, oxygen transport, and deoxyribonucleic acid (DNA) synthesis. However, iron deficiency (ID) and its associated anaemia (IDA) are major contributors to the global burden of disease, affecting up to one-third of the world‘s population (Man, Xu, Adhikari, Zhou, Wang, & Wang, 2021). Out of the addressable multifactorial causes underlying ID, nutritional deficiency from low dietary intake and/or poor absorption from dietary sources are amongst the most frequent (Camaschella, 2015).

Orally administered iron can be obtained through a wide range of traditional food sources, as well as supplemental forms such as mineral salts. Nevertheless, the efficiency of dietary iron absorption from most sources can be low, with reports ranging from 5-20% of absorption in a mixed diet (Schulze & Dreyfuss, 2005). Nonheme iron from vegetal sources can form insoluble complexes with other anions at the major iron absorption site of the duodenum, impeding iron transportation across the intestinal enterocytes (Sun et al., 2020). Heme iron from animal tissue is more bioavailable owing to its vehiculation by a protoporphyrin ring ligand. It is however less accessible to populations lacking access to animal-derived food products, whilst independently posing health risks such as cancers

(Man et al., 2021). Conventional supplemental forms of iron salts also have accessibility issues, as well as being commonly linked to deleterious side effects associated with poor bioavailability (Caetano-Silva, Cilla, Bertoldo-Pacheco, Netto, & Alegría, 2018).

Recent evidence has suggested that the most well-tolerated form of oral iron supplement is an Fe(III)-complex formed with succinylated casein (Cancelo-Hidalgo et al., 2013). As such, food protein-derived peptides have been closely explored as alternative oral carriers of iron to enhance its bioavailability. Encrypted in diverse proteins, these peptides may reduce ferric iron to improve its solubility, or chelate iron by forming a complex with its amino acid side chains through coordination and ionic bonds principally mediated through the -NH_2, -COOH and -SH moieties (Man et al., 2021). Numerous iron-binding peptides have been generated using enzymatic degradation from a number of food proteins, including milk, krill, chickpea, and mung bean (Wu, Yang, Sun, Bao, & Lin, 2020). The enzymatic hydrolysates of pea proteins (*Pisum sativum L.*) are known to have iron-chelation property (Pownall, Udenigwe, & Aluko, 2010), and have been characterized and studied for other food applications. However, its relevance towards iron bioaccessibility enhancement remain underexplored.

Pea proteins possess a broad proteomic diversity, low allergenicity and sustainable broad-scale production traits (Daba & Morris, 2022). During biological development, a wide array of iron-binding proteins mediating biological iron acquisition and storage such as ferritin (Moore et al., 2018), as well as stress tolerance such as hydrophilins (Battaglia & Covarrubias, 2013) accumulate in the seed, which can eventuate as rich sources of iron-binding motifs. Our previous investigation found that pea protein hydrolysates produced

from a combination of phytase and protease treatments was able to enhance the *in vitro* small intestinal bioaccessibility of iron supply as $FeCl_3$ at a concentration of 15 mM (Zhang, Stockmann, Ng, & Ajlouni, 2022). We attributed this effect to an increase in iron-solubilizing peptides, due to the concomitant observation of an increase in soluble protein and degree of hydrolysis. Subsequently, the objectives of the current study were to: 1) determine the iron(III)-binding properties of the pea hydrolysate; 2) separate the iron-binding peptides; 3) characterize the structural transformation in pea hydrolysate-iron(III) complexes; and 4) identify the peptides potentially contributing to the enhancement of iron(III) solubility, hence bioaccessibility.

6.2. Materials & Methods

6.2.1. General reagents and equipment

Common chemicals and consumables were of analytical reagent grade or better and were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). IDA-Sepharose resin (Cytiva Life Sciences, Chelating Sepharose Fast Flow 6B, cat no. 17057504), C18 Spin Columns (Pierce™ C18, cat no. 89870) and sequencing-grade trypsin (Promega, cat no. V5111) were acquired from Thermo-Fisher Scientific (Scoresby, Victoria, Australia). Pea protein isolate (PPI, 82% protein) from golden field peas was acquired from Pure-Product Australia (Mascot, New South Wales, Australia). Protease M 'Amano' (protease peptidase from *Aspergillus oryzae, activity* 10,000 U/g) was kindly provided by Wilmar Bioethanol (North Sydney, New South Wales, Australia), and wheat phytase (6-phytase, activity 50 U/g) was purchased from Sigma-Aldrich. Lactoferrin (96.6% purity, 14% iron saturation) was kindly gifted from Dr. Bradley Coleman of Bega Bionutrients (Port Melbourne, Victoria, Australia). All reagents were made with deionised water (≥18

MΩ) produced in-house using a Synergy UV Millipore System (Merck Millipore, Victoria, Australia).

6.2.2. Enzymatic hydrolysis of pea protein isolate

The enzymatic hydrolysis was performed in two steps following the method of Zhang et al. (2022). Firstly, a reaction mixture consisting of the PPI (100 mg/mL) and phytase at 0.8 U/g of the solids equivalent, was incubated for 3 hours at 50 °C (ZWYR-240, Labwit Scientific, Shanghai, China), with the pH maintained at 5.0 every hour using 1 M HCl/NaOH. Protease M was then added to the mixture at 400 U/g of solids equivalent as recommended by the manufacturer, and was maintained under the same incubation conditions for an additional 3 hours, prior to the inactivation of enzymes by bringing the pH to 10 using 1 M NaOH. The hydrolysates were recovered by adjusting the pH back to 5.0, prior to centrifugation at 2000 *x g* for 15 min (Allegra X-12R, Beckman Coulter, Land Cove West, New South Wales, Australia). The pellets were frozen at −20°C and lyophilized.

6.2.3. Determination of Fe(III)-binding capacity

The Fe-binding capacity of the enzymatic hydrolysate was examined by the methods of Kim et al. (2007) with some modifications. The lyophilized hydrolysates were reconstituted to 1% w/v with the addition of 0.1% $FeCl_3$ to a final volume of 10 mL and the pH adjusted to 5.0. A positive control (lactoferrin) was also prepared. All samples were incubated at 37°C for 1 h and centrifuged at 1500 *x g* at RT (21 °C) for 5 mins. The soluble (supernatant) and total Fe in each of the solutions were then measured following the method of Zhang et al. (2022) using inductively-coupled plasma spectroscopy (Optima

8300 DV ICP-OES, Perkin Elmer, Glen Waverley, Australia). Iron-binding was calculated as follows:

$$Iron\ binding\ capacity\ (mg) = \frac{Fe_{soluble}}{Fe_{total}}$$

where $Fe_{soluble}$ is the supernatant iron concentration from the sample suspension and Fe_{total} is the total iron concentration of the sample suspension. Replicate samples (n = 3) were determined and uncertainty of the mean was expressed as standard deviation (mean ± SD).

6.2.4. Immobilised-metal affinity chromatography (IMAC) separation of Fe(III)-binding peptides

IMAC separation of the iron-binding peptides from the enzymatic hydrolysate was conducted following a modified method of Lv, Bao, Liu, Ren, and Guo (2013), adapted to a benchtop pump. A mini chromatography column (Cellufine, JNC Corporation, Tokyo, Japan) was packed with 1 mL of IDA-Sepharose 6B resin and charged with three bed volumes of 15 mM iron as $FeCl_3$. The resin was then washed with five bed volumes of deionised water to remove unbound iron, and another five bed volumes of sodium acetate-acetic buffer (50 mM, pH 4.0) to remove non-specific bound iron. The column was equilibrated with HEPES buffer (20 mM, pH 8.0) containing 100 mM NaCl before loading the reconstituted hydrolysate (3 mL containing 10 mg/mL lyophilized solids) onto the column. Peptides with low affinity for immobilised iron were eluted with the equilibrating buffer and collected as the non-adsorbed fraction. The bound peptides were eluted with salt gradient elution buffers containing 100 mM $NH_4H_2PO_4$ (pH 6.0, 150-600 mM NaCl), and collected for lyophilization for use in further analyses.

6.2.5. Fourier-transformed infrared spectroscopic analysis (FTIR)

Fe(III)-peptide complexes were prepared by slowly adding 0.5 mL of 10 mM iron as $FeCl_3$ solution (0.279 mg Fe) to a 10 mL solution of the IMAC-separated peptide solution (10 mg/mL, total of 100 mg solids) that had undergone secondary elution through an uncharged IMAC column to remove excess Fe ions. The mixture was shaken periodically at RT (21 °C) for 30 min to allow complex formation. Solutions of the iron-peptides complex mixture, the $FeCl_3$ solution, and the peptides (10 mg/mL) without $FeCl_3$ were then subjected to FTIR analyses. The IMAC phosphate elution buffer was also examined in both the presence and absence of $FeCl_3$ to exclude phosphate interaction effects.

The FTIR samples were analysed with a Nicolet™ iS50 Continuμm™ Infrared (IR) microscope (Thermo Fisher Scientific, Waltham, USA) equipped with a mercury cadmium telluride detector. Samples were spread as thin hydrated films over an uncoated BaF_2 window with 12.5 mm diameter x 2 mm thickness (Edmund Optics, Singapore). The IR objective was magnified 32 times with a spectral resolution of 4 cm^{-1}. The IR spectra was collected between 4000 and 700 cm^{-1} in transmittance mode, with 30 scans per sample. Baseline corrections were performed by subtracting the background spectrum from the BaF_2 window collected prior to each analysis, using the OMNIC 8.2 software (Thermo-Fisher Scientific, Scoresby, Victoria, Australia). The method was validated by examining 250 mg of Bovine Serum Albumin (BSA) on the BaF_2 window, and identifying the major characteristic peaks at ~3500 and ~1650 cm^{-1} as the O-H and C=O stretches from amides A and I, respectively (Alhazmi, 2019).

Interpretation and graphing of the spectra were conducted on OriginPro 2021b (OriginLab Corp., Northampton, USA). Spectral smoothing was performed using a second derivative originally described by Savitzky and Golay (1964) (nine smoothing points, polynomial order 2). Interpretations of the vibrational frequencies are based on that reported by Yang, Yang, Kong, Dong, and Yu (2015).

6.2.6. Identification of peptides using Liquid Chromatography Mass Spectrometry

6.2.6.1. Sample preparation

The lyophilized peptide fraction from IMAC was solubilized in 150 µL of 0.1% formic acid and processed using a Pierce C18 Spin Column according to the manufacturer's instructions, with the following modifications: the trifluoroacetic acid (TFA) was substituted with formic acid, and the sample was gently vortexed after addition to the filter and allowed to incubate at RT (21 °C) for 1 min. The resultant solution was divided into 2 aliquots in separate tubes before removal of water by rotary evaporation and the residue stored at -20°C.

In-solution tryptic digestion was performed on one of the sample aliquots (dPH-Fe) to ensure that peptides were amenable to mass spectrometric analysis. The residue was solubilised in 25 µL of 50 mM NH_4HCO_3 containing 5 mM DTT at 300 rpm in a Thermomixer (Eppendorf, Macquarie Park, New South Wales, Australia) and incubated at 50°C for 30 min. To alkylate the cysteine residues, iodoacetamide (100 mM, 2.5 µL) was added to the digest and incubated for 30 min in the dark at RT (21 °C) with continuous shaking at 300 rpm. Sequencing grade porcine trypsin was added (2.5 µL, 1mg/mL in

50mM NH_4HCO_3) to the samples and incubated for 16 h at 37˚C in a Thermomixer at 300 rpm. The sample was acidified after digestion by the addition of 11.5 µL of 1% formic acid. The undigested sample (PHFe) was solubilized directly in 0.1% formic acid (15 µL) with sonication for 5 min at RT (21 °C).

6.2.6.2. LC-MS analysis

Peptides were analyzed with chromatographic separation on an Ekspert nanoLC415 (Eksigent, Dublin, CA, USA) directly coupled to the DuoSpray ion source of a TripleTOF 6600 LC-MS/MS (SCIEX, Redwood City, California, USA). The peptides were desalted for 5 min on a Trajan ProteCol C18 (3 µm, 120 Å, 10 mm × 0.3 mm) trap column at a flow rate of 10 µL/min 0.1% formic acid (FA), and separated on an Eksigent C18 (3 µm, 120 Å, 150 mm × 0.3 mm) column at a flow rate of 5 µL/min maintained at 30°C (Bose et al., 2021). A linear gradient was applied using two solvents, (A) aqueous solution of 0.1% formic acid / 5% DMSO (FA) and (B) aqueous 0.1% FA / 5% DMSO in 90% acetonitrile. The linear gradient employed 2-30% solvent B (90 min), 30-50% B (10 min), 50-80% B (5 min), at 80% B (5 min), 80-98% B (1 min), at 98% B (4 min), 98-2% B (1 min) and re-equilibration at 2% (B14 min).

The instrument parameters were as follows: ion spray voltage 5500 V, curtain gas 25 psi (equiv. 172368.932 pa), GS1 13 psi (equiv. 89631.8448 pa) and GS2 15 psi (equiv. 103421.359 pa), heated interface 150°C. Data were acquired in information-dependent acquisition (IDA) mode comprising a time-of-flight (TOF)-MS survey scan followed by 30 MS/MS product ion scans. First stage MS analysis was performed in positive ion mode, mass range m/z 350−1400 and 0.25 s accumulation time. Tandem mass spectra were

acquired on precursor ions >150 counts/s with charge state 1−5 and dynamic exclusion for six s after one occurrence with a 50 ppm mass tolerance. Dynamic background subtraction was selected to minimize acquisition of +1 charge background ions.

6.2.6.3. Protein Identification

Tandem mass spectrometry data was searched against *in silico* tryptic digests of custom-built databases. The databases comprised the proteins of *Pisum sativum*, appended with a database of contaminant proteins (known as the common repository of adventitious proteins, cRAP) and internal retention time standards (iRT). The search parameters were defined as: thorough; iodoacetamide modified for cysteine alkylation and trypsin as the digestion enzyme for the digested sample only. A custom instrument profile was used to consider the acquisition +1 charge state peptide ions during signal processing and searching. Additional modifications and cleavages were defined previously (Colgrave, Goswami, Blundell, Howitt, & Tanner, 2014). The database search results were manually curated to yield the protein identifications using a 1% global false discovery rate (FDR) determined by the ProteinPilot software companion FDR tool (Tang, Shilov, & Seymour, 2008).

6.2.7. Physicochemical properties and bioinformatics analysis

6.2.7.1. Extraction of peptide features

The protein sequence files in *.FASTA format were created for the full list of peptides identified from dPH-Fe. The sequence statistics were then analyzed in MATLAB R2022b (MathWorks, Inc., Massachusetts, USA). Functions within the Bioinformatics

Toolbox were used to determine the amino acid counts, net charge (pH 7.0) and isoelectric point (pI) for each peptide. Additionally, the average hydrophilicity for each peptide was processed using a user-defined script based on the hydrophilicity values of Hopp and Woods (1981).

6.2.7.2. Screening of candidates for enhancing Fe(III) bioaccessibility

To identify the major peptides that may be used to enhance Fe(III) solubility during human small intestinal digestion, the list of peptides identified from dPH-Fe was refined based on the following criteria. The peptides were firstly analyzed using the AllerCatPro 2.0 web server (*https://allercatpro.bii.a-star.edu.sg/*) to investigate their putative allergenicity, eliminating peptides with any evidence of % identity to a confirmed 3D epitope of a known allergen. The peptides were then filtered based on molecular weight and relative abundance, retaining those with <1.5 kDa and sequence abundance >10%. This criterion was based on the consensus that most food-derived peptides with high Fe-binding affinity have a range between 0.7 – 1.5 kDa (Wu et al., 2020). The remaining peptides were analyzed and ranked based on their chelation score using AnOxPePred-1.0 (*https://services.healthtech.dtu.dk/service.php?AnOxPePred-1.0*), a deep-learning computational tool that can assign a score between 0-1 based on its predicted chelation properties. Finally, peptides with a pI above intestinal conditions (pH 6.5) were excluded due to likelihood of precipitation or electrostatic repulsion with Fe(III).

6.2.7.3. Detection and distribution of potential Fe(III)-binding motifs

Due to the lack of a reference dataset for Fe(III)-binding peptides in *P. sativum*, the motifs overrepresented in the dPH-Fe fraction was identified *ad hoc* using the *nmercount* function in the Bioinformatics Toolbox on MATLAB. Considering that the intestinal bioavailability of compounds decreases rapidly with MW > 700 Da, a minimum of three, and maximum of six amino acid residues were included in the motif search, assuming an average monoisotopic MW of 110 Da per amino acid (i.e. a maximum of 660 Da). The top 200 motifs of each sequence length (n-mers: 3-6) were assembled by absolute abundance, where an approximate Lorenz curve was generated for each sequence length to map its distribution. This was performed using the *concentrationIndices* function in the Risk Management Toolbox on MATLAB. The most abundant motif for each n-mer was also analyzed for the chelation score using AnOxPePred-1.0.

6.3. Results

6.3.1. Iron-binding capacity of the hydrolysate

The soluble Fe content in the presence of both the pea hydrolysate and $FeCl_3$ as a fraction of the total Fe present was measured as a proxy for bioaccessible ferric iron [Fe(III)]. The sample containing 100 mg pea hydrolysates had a binding capacity of 0.53 ± 0.09 mg soluble iron in solution at pH 5, equivalent to 5.3 mg iron bound per gram of powder. In comparison, the same quantity (100 mg) of lactoferrin solubilized 0.99 ± 0.05 mg Fe(III), equivalent to 9.9 mg bound Fe per gram of powder.

6.3.2. FTIR Characterization of the Fe(III)-hydrolysate complex

FTIR is frequently used for conformational analysis or structural characterization of peptides, where the formation of Fe-peptide complexes can lead to shifts in, gain, or loss of certain absorbance peaks. As shown in **Figure 16**, spectrums from hydrated films of the hydrolysate-Fe complex suggested some conformational differences of the peptides in the presence of Fe(III) ions in both the fingerprint and functional regions. The appearance of new peaks at 1710 cm^{-1} and 1460 cm^{-1} after the addition of iron could be respectively assigned to the stretching vibration of the carboxyl groups (COO−), and Amide II (C-N and in plane N-H bending) that played a role in iron binding. A band corresponding to the Amide I group was shifted to lower frequency from 1620 to 1610 cm^{-1}, which is associated with mainly the C=O stretching vibration and the backbone conformation. Additionally, the original peak at 876 cm^{-1} from the PH-Fe was split into two peaks at 845 and 899 cm^{-1}, which likely corresponded to iron binding by carboxylate groups. Some Fe(III)-phosphate interactions can be observed in the spectrums of the IMAC elution buffer containing phosphate with and without Fe(III) ions (*Supplementary Data 2*). However, the frequency shifts occurred at different ranges (979 – 1031 and 2983 – 3086 cm^{-1}) and thus are unlikely to account for the interactions observed between the peptide hydrolysates and iron.

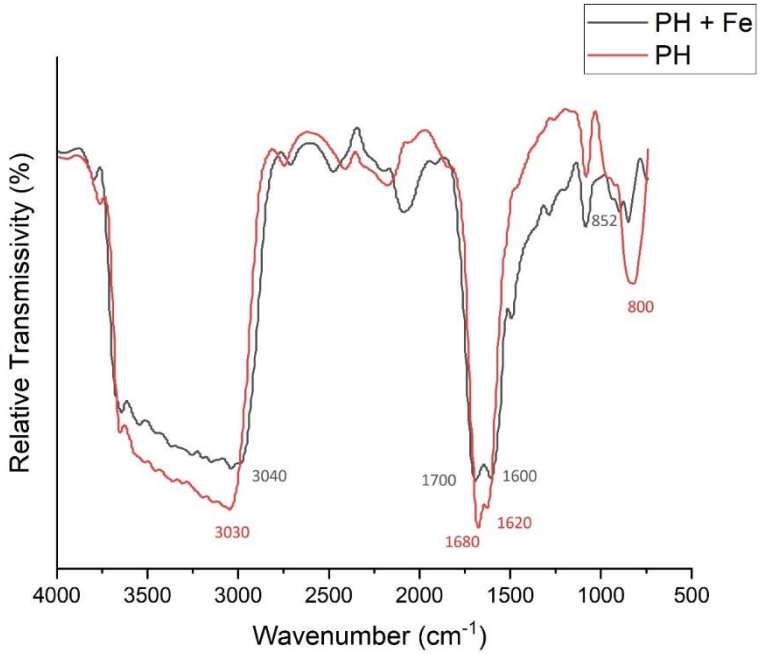


Figure 16. Fourier transform infrared (FTIR) spectra of pea protein hydrolysates (PH) and pea hydrolysate-iron complexes (PH + Fe) in the regions from 500 to 4,000 cm^{-1}.

Table 16. Protein assignment of the peptides identified in the dPH-Fe fraction

Origin	**% of total**
Classified by accession only	24.48
Q9M3X6_PEA Convicilin	16.77
B0BCJ4_PEA Convicilin (Fragment)	16.22
VCLC_PEA Vicilin	14.97
Q702P1_PEA Vicilin (Fragment)	14.50
VCLA_PEA Provicilin (Fragment)	5.88
LEGA2_PEA Legumin A2	3.64
LEGJ_PEA Legumin J	1.40
O49927_PEA p54 protein	1.32
ALB2_PEA Albumin-2	0.78
LOX3_PEA Seed linoleate 9S-lipoxygenase-3	0.03

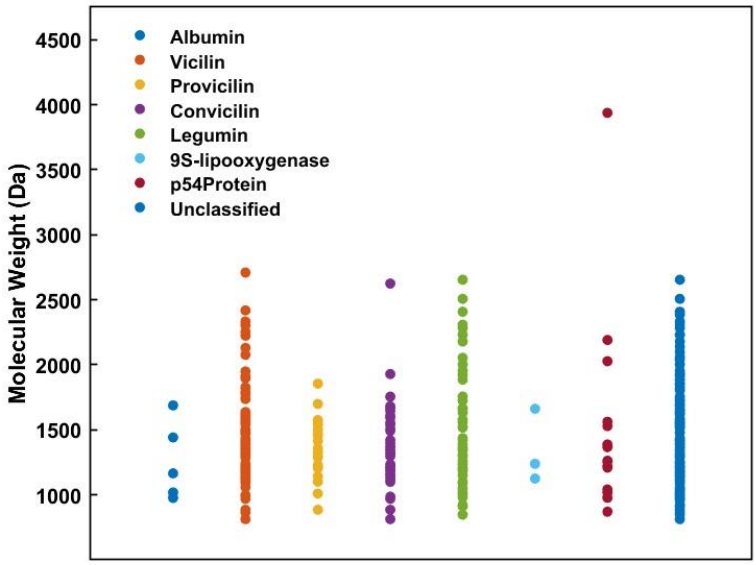
6.3.3. Global features of the Iron-Binding peptides

6.3.3.1. Sequence analysis by LC-MS and protein classification

Sequence matching of the PH-Fe and dPH-Fe showed a similar number of proteins found for these two fractions. In total, peptide evidence supported the identification of 38 proteins that were detected from the PH-Fe fraction, and 29 from the dPH-Fe at a global critical false discovery rate of 1%. In consonance with the role of iron in cellular biology, there was a broad distribution of iron-binding proteins throughout different functional classes. Whilst not all proteins were functionally annotated in the custom-built database, the majority of the existing identifications stem from the seed storage globulins Vicilin, Convicilin and Provicilin (**Table 16**). Another major iron-binding protein identified was the O49927_PEA p54 protein, a class of highly charged, hydrophilic nuclear proteins with cupin-like and intrinsically disordered domains.

The dPH-Fe fraction containing 277 peptides was subjected to further analysis of their physicochemical properties within MATLAB. The analysis revealed that the molecular weight (MW) of the iron-binding peptides ranged from 813 kDa to 3937 kDa (**Figure 17-a**). As demonstrated in **Figure 17-b**, the most abundant amino acid in the iron-binding peptides was glutamic acid (n = 625), followed by asparagine, lysine and leucine (n = 247, 245 and 236, respectively). The dPH-Fe fraction also showed low levels of cysteine, methionine, and tryptophan residues (n = 9, 3 and 11, respectively).

a)



b)

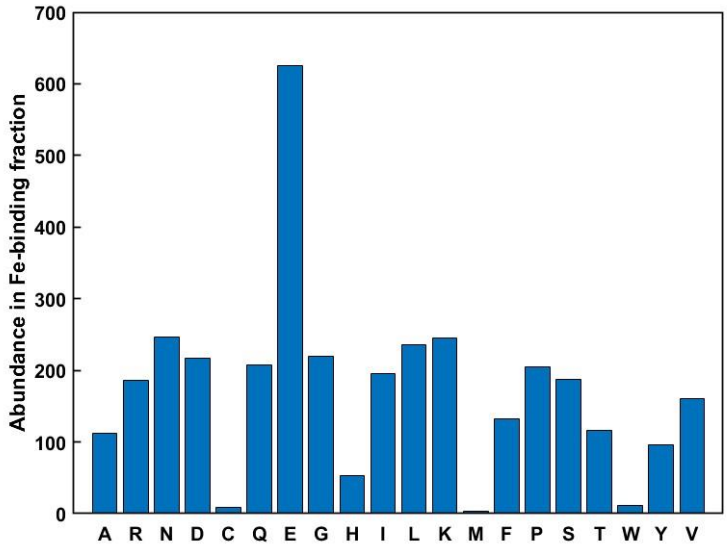


Figure 17. Characteristics of the peptides identified in the d-FePH fraction, showing a) the molecular weight (MW) distribution of peptides from different classes, and b) absolute abundance of the various amino acids in the peptides identified. A: Alanine; R: Arginine, N: Asparagine; D: Aspartic acid, C: Cysteine, Q: Glutamine; E: Glutamic acid; G: Glycine; H: Histidine, I: Isoleucine, L: Leucine; K: Lysine; M: Methionine; F: Phenylalanine, P: Proline; S: Serine; T: Threonine; W: Tryptophan, Y: Tyrosine; V: Valine.

6.3.3.2. Identification of potential Fe(III)-binding motifs

Following the *ad hoc* identification of potential motifs based on their absolute abundance in dPH-Fe, the inequality amongst the distribution was graphically represented using the Lorenz curve (*Supplementary Data 2*). The curve generated for each sequence length showed that for the sequence lengths of 3, 4, 5, and 6, the top 2 deciles composed 42%, 41%, 39% and 36% of the total abundance in each of the n-mers, respectively. The top 2 deciles are presented in **Table 17**, which demonstrated the presence of multiple consecutive Glu residues up to 6-mers. Input of the top motif for each n-mer into AnOxPePred-1.0 showed chelation scores of 0.24871, 0.24825, 0.23718 and 0.26892 for 'EEE', 'EEEE','EEEEE' and 'DEEEEQ', respectively. These are comparative to the score generated by the di-peptide GG (0.27707), which forms the bisglycinate commonly found in iron supplements (Cancelo-Hidalgo et al., 2013).

Table 17. The sequence motifs identified ad hoc for each n-mer (3-6) in the top 2 deciles of the Lorenz Curve, based on their number of occurrences (n) within the d-FePH fraction

n-mer = 3		*n-mer = 4*		*n-mer = 5*		*n-mer = 6*	
	n		*n*		*n*		*n*
'EEE'	109	'EEEE'	57	'EEEEE'	25	'DEEEEQ'	14
'EDE'	42	'DEDE'	19	'DEEEE'	18	'EEEEQG'	13
'DEE'	38	'DEEE'	19	'EDEDE'	17	'EEEQGE'	13
'EEQ'	25	'EEEQ'	18	'EEEEQ'	17	'EEQGEE'	13
'EED'	23	'EDED'	17	'QGEEE'	14	'EQGEEE'	13
'EEI'	23	'GEEE'	17	'EEEQG'	13	'EEEINK'	12
'GEE'	20	'EDEE'	16	'EEQGE'	13	'GEEEIN'	12
'DED'	19	'EEDE'	16	'EQGEE'	13	'IEELSK'	12
'QGE'	19	'EQGE'	16	'EEEIN'	12	'QGEEEI'	12
'DDE'	17	'QGEE'	16	'EEINK'	12	'QIEELS'	12
'QEE'	17	'DDEE'	15	'EELSK'	12	'EDEDEE'	11
'EQG'	16	'EEQG'	13	'GEEEI'	12	'DDEEEE'	10
'EIN'	15	'EEEI'	12	'IEELS'	12	'EDDEEE'	10
'EEL'	13	'EEIN'	12	'QIEEL'	12	'EEEEEE'	9

'LSK'	13	'EELS'	12	'DEDEE'	11	'KEDDEE'	9
'VIV'	13	'EINK'	12	'DDEEE'	10	'YEEIEK'	9
'YEE'	13	'ELSK'	12	'EDDEE'	10	'EEDEDE'	7
'EIE'	12	'IEEL'	12	'GDTIK'	10	'EEINKQ'	7
'ELS'	12	'QIEE'	12	'NVIVK'	10	'FENLQN'	7
'IEE'	12	'DTIK'	10	'EEIEK'	9	'DEDEDE'	6
'INK'	12	'EDDE'	10	'KEDDE'	9	'DTIKLP'	6
'IVK'	12	'GDTI'	10	'YEEIE'	9	'EDEDED'	6
'QIE'	12	'NVIV'	10	'QEEDE'	8	'EENVIV'	6
'YSN'	12	'VIVK'	10	'EEDED'	7	'ENLQNY'	6
'EKN'	11	'EEIE'	9	'EINKQ'	7	'ENVIVK'	6
'NKQ'	11	'EIEK'	9	'ENLQN'	7	'ETWNPN'	6
'PAG'	11	'KEDD'	9	'FENLQ'	7	'GDTIKL'	6
'PET'	11	'SNKF'	9	'IKLPA'	7	'IKLPAG'	6
'SNK'	11	'YEEI'	9	'PIYSN'	7	'NLQNYR'	6
'DTI'	10	'IPVN'	8	'QQSQE'	7	'PGCPET'	6
'EDD'	10	'QEED'	8	'TWNPN'	7	'TIKLPA'	6
'GDT'	10	'EDLR'	7	'DEDED'	6	'DDEEDL'	5
'KED'	10	'EEED'	7	'DTIKL'	6	'DEEDLR'	5
'NPE'	10	'ENLQ'	7	'EEEDE'	6	'DLAIPV'	5
'NPF'	10	'FENL'	7	'EEEED'	6	'DYEEIE'	5
'NVI'	10	'IKLP'	7	'EENVI'	6	'EEEEDE'	5
'TIK'	10	'INKQ'	7	'ENVIV'	6	'FFEITP'	5
'EQE'	9	'IYSN'	7	'ETWNP'	6	'KLPAGT'	5
'FEN'	9	'KLPA'	7	'FEITP'	6	'KQIEEL'	5

6.3.4. Identification of candidates for Fe(III) bioaccessibility enhancement

Data in **Table 18** present the list of candidates identified from dPH-Fe that have the potential to enhance Fe(III) solubility during small intestinal digestion. The total of 15 peptides reflected the narrow specificity of cleavages by trypsin, with eight containing arginine, and seven with lysine at the C-terminal. All peptides demonstrated >50% of hydrophilic versus overall residues, with two peptides (QEEDEDEDEER and QEEEEDEDEER) exhibiting 100% hydrophilic residues. These two highly hydrophilic peptides were classified as Legumins. The other peptides identified stem primarily from the Convicilin or Vicilin family, except for LDALEPDNR and EEEAEAEAR that are unclassified. The range of chelation scores computed for the list ranged from 0.18031 (FNTNYEEIEK) to 0.28043 (SDLFENLQNYR).

Table 18. Sequences of the candidate peptides selected from the d-FePH fraction. The MW and m/z are observed values, and the chelation score was computed by AnOxPePred 1.0.

Sequence	Molecular Weight (Da)	Mass-to-charge (m/z)	Chelation Score	pI	Net charge (pH 7.0)	Average hydrophilicity
SDLFENLQNYR	1397.64	699.83	0.28043	4.09	-1	0.14
LDALEPDNR	1041.50	521.76	0.26413	3.64	-2	0.9
FENENGHIR	1114.50	558.26	0.23788	5.31	-0.91	0.51
VLLEEQEK	986.52	494.26	0.23711	3.95	-2	0.89
EEEAEAEAR	1032.42	517.22	0.23185	3.54	-3.99	1.83
QEEDEDEDEER	1421.49	711.75	0.2284	3.15	-7.99	2.75
YENENGHIR	1130.50	566.26	0.22489	5.31	-0.91	0.53
QEEEEDEDEER	1435.52	718.76	0.22449	3.18	-7.99	2.75
REQIEELSK	1130.58	566.30	0.22263	4.63	-1	1.32
NQDDEEDLR	1132.45	567.23	0.21395	3.33	-4	1.84
QEINEENVIVK	1313.67	657.84	0.20902	3.95	-2	0.55
QQSQEENVIVK	1299.69	434.24	0.20349	4.3	-1	0.48
NTDYEEIEK	1139.49	570.75	0.19527	3.58	-3	1.19
FNTDYEEIEK	1286.56	644.29	0.19118	3.58	-3	0.82
FNTNYEEIEK	1285.58	643.80	0.18031	3.95	-2	0.54

6.4. Discussion

Most dietary iron enters the small intestinal tract as Fe(III), the thermodynamically favoured species under aerobic milieus. However, the species generated following its dissolution ($[Fe(H_2O)_6]^{3+}$) rapidly undergoes hydrolytic polymerization and precipitation as Fe(III) hydroxides or hydrated Fe oxides (Sánchez, Sabio, Gálvez, Capdevila, & Dominguez-Vera, 2017). Subsequently, higher concentrations of soluble Fe(III) ($> 10^{-18}$ M) is only achieved through complexation with low MW ligands such as peptides/amino acids, organic acids and sugars. This study aimed to characterise the pea peptides that not only possess the right electronic and steric configuration in binding Fe(III), but also may exhibit the desired binding behaviour under the dynamic conditions (i.e. solubility, ionic strength and pH) observed during human digestion. Such peptide-ferric complexes should garner sufficient affinity to remain intact throughout gastric digestion to prevent iron release, whilst avoiding excess metal-peptide aggregation under the highly ionic environment of the small intestine.

The iron-binding capacity of peptic hydrolysates is a quantitative interplay between the protein precursor, iron form (ferrous or ferric), enzyme type and hydrolysis time, and the values obtained are dependent on the analytical method. Accordingly, the pea hydrolysate in this study showed Fe(III) binding capacity of 5.3 mg/g, which is comparable to those reported in soybean peptides (4.72 mg/g) using atomic absorption spectrometry (Liu, Bao, Lv, Xu, & Guo, 2012). However, the herein reported iron-binding capacity of 5.3 mg/g pea protein was significantly smaller than those obtained in soy and mung bean hydrolysates (36.81 and 47.75 mg/g, respectively), when colorimetric displacement

methods were used (Chunkao, Youravong, Yupanqui, Alashi, & Aluko, 2020; Giarni, Saepudin, Susanti, & Laily, 2020). Such variations could be attributed to the fact that hydrolysates used in this iron-binding study were not purified, as we aimed to characterize the original carrier employed in our previous *in vitro* digestion investigation (Zhang et al., 2022). As such, the impure hydrolysates may contain other counterions that can promote solubilization of Fe(III) and prevent aggregation between peptides and Fe(III). Examples of such counterions are the negatively charged phosphates released from phytase hydrolysis and soluble carbohydrates (Carbonaro, Maselli, & Nucara, 2015). However, the chelation property of the pea hydrolysate would likely be higher with the purified peptide fraction (Sun et al., 2020), and if all the endogenous iron had been removed. Similarly, whilst our results suggest that lactoferrin has approximately twice the binding capacity relative to the pea hydrolysate, it was noted also that the lactoferrin binding values obtained were from a partially iron-saturated lactoferrin, and thus expected to have a lower binding power than iron-free (apo) lactoferrin.

The MW of iron-binding peptides is a crucial factor affecting both complexation and stability properties. The transportability of peptides generally decreases with size regardless of the trans- or paracellular route, unless the internalization is mediated by specific receptors (Sun et al., 2020). We employed a cut-off of 1.5 kDa, based on the current knowledge that peptide-metal complexes are absorbed similarly to the transport pathway as regular peptides (Sun et al., 2020). Previously sequenced iron-binding food peptides have mostly been <1.5 kDa, a feature corroborated by research identifying the lower MW fraction of other peptide hydrolysates (<1 kDa) to be superior iron chelators

than higher MW fractions (10 kDa) (Xia, Bamdad, Gänzle, & Chen, 2012). Similar trends have been reported in pea, where peptides with a MW <3 kDa reportedly have better Fe-chelating activity versus the crude hydrolysates (Pownall, Udenigwe, & Aluko, 2010). According to Caetano-Silva et al. (2018), whilst peptides up to 5 kDa may still demonstrate high solubility of the iron complex *in vitro*, the cellular uptake can be significantly lower than that within the sub-5 kDa fraction. This suggests that complexes with lower MW may be necessary, at least for the higher bioavailability transcellular route of absorption (Wang & Li, 2017).

The distribution characteristics of amino acids in the dPH-Fe fraction suggests that some modes involved in binding may be specific to Fe(III). Generally, iron binding peptides are commonly linked to the abundance of charged amino acid residues such as Asp, Glu, Arg and His. Whilst previous studies have identified His to play an important role in binding iron, it was the fourth least abundant amino acid in dPH-Fe. This could be partially explained by the lower His content of pea (~2% of proteins) (Gorissen et al., 2018), as well as the absence of imidazole in the IMAC elution buffer. However, Fe(III) also has a selective specificity towards 'hard' oxyanions (e.g. phenolate and carboxylic acids), which differs from Fe(II) that prefers N- and S- ligands (e.g. imidazole N-donors from His). This correlates with the FTIR spectra of PH-Fe in the presence of Fe(III), which showed that the main mode of complexation was through carboxyl oxygen along with amide binding. The same chief binding modes have been reported from other Fe(III)-binding peptides isolated from hydrolysates of casein, β-Lg, and shrimp processing by-products (Wu et al., 2020). Results from this current study also found low levels of

aromatic and sulphur amino acids in dPH-Fe, which was expected as they are limited in legumes. Similarly, low concentrations of Cys and His have also been reported in other studies of food peptide isolates using Fe(III), including soy and meat tissues (Lv, Liu, Bao, Tang, Yang, & Guo, 2009; Storcksdieck, Bonsmann, & Hurrell, 2007). However, the opposite has been observed in peptides from Pacific cod skin, when ferrous ion (Fe(II)) was used (Wu, Li, Hou, Zhang, & Zhao, 2017).

Many of the peptides in dPH-Fe echo the properties of legume proteins that are highly soluble at near neutral pH (6.5), which is above the pKa of most peptides that have been identified to be responsible for Fe(III) binding in pea protein hydrolysate. These are characterized by lower levels of hydrophobic amino acids, rich in Glu and Arg residues, and are highly charged (Carbonaro, Cappelloni, Nicoli, Lucarini, & Carnovale, 1997). Although pea proteins are generally high in Glu, its occurrence in dPH-Fe exceeded 200-fold to the least abundant (Met). The role of Glu in Fe(III)-binding peptides has been established in several studies, notably by Hu, Lin, Wang, Zhang, and Sun (2021) who showed that Fe(III)-binding capacity is directly linked to both the amount and positioning (consecutive or alternating) of Glu in hydrolysates of Antarctic krill. Glu has an acidic side chain that is deprotonated at intestinal pH (pKa 4.4), where Fe(III) binding takes place at the free γ-and δ-carboxyl groups to form stable ferric chelates (Storcksdieck et al., 2007). Considering that Glu also has an aqueous solubility advantage over other hydrophilic amino acids (Trevino, Scholtz, & Pace, 2007), an appropriate ion-to-residue ratio with free coordination sites for water would assist to minimize peptide aggregation during intestinal digestion. In the peptide list identified from this study, 10 followed the sequence Glu-X-X-Glu, a recurring ferric-binding motif in several biological proteins involved in iron sensing, transport and storage (Fang & Wang, 2002). Similarly, the top motifs identified *ad hoc* in this study also consisted of consecutive Glu residues. These may be further explored as Fe(III)-binding tri-, tetra- and oligopeptides, especially given their comparable chelation scores to the peptide candidates found in this study.

Recent advancement towards identifying individual food iron-binding peptides has been indispensable to the understanding of their structure-activity relationships, which can further be used in strategies to enhance oral iron bioavailability. However, care must be taken to achieve a moderate binding affinity that facilitates eventual dissociation (Sun et al., 2020). Whilst separation and purification of the peptide can lead to higher iron-chelating activity, strong binding can reduce or eliminate the solubility of the peptides. Such event may be mitigated by designing structures that notably act on synergisms that 1) promote complex solubility and 2) minimize its reactivity in gastrointestinal environments. This is illustrated by the synthesis of a novel iron(III)-casein complex, which originally forms an insoluble precipitate at equimolar ratios with iron, but is resolubilized by the adsorption of orthophosphates (Mittal, Ellis, Ye, Edwards, & Singh, 2018). In the context of pea proteins, glycated pea proteins can exhibit higher susceptibility to protease hydrolysis (Świątecka, Kostyra, & Świątecki, 2010), thereby enhancing the release of soluble iron-binding peptides within the gut.

Most of the iron-binding peptides in PH-Fe were found to be vicilins, which garnered insights towards the nature of iron bioaccessibility in relation to peas. The 7S Globulins are a major storage protein in legumes, and thus an unsurprising parent source. However, 7S proteins are known to possess structural characteristics that lead to resistance by intestinal enzymes, even after heating (Tavano & Neves, 2008). This indicates that under conditions where conventional pea proteins are delivered with Fe(III) (e.g. endogenous or biofortified seed Fe, and supplementation of Fe with meals), there is likely a fraction bound inside undigested 7S proteins limiting its bioaccessibility. The relationship between 7S proteins and iron has also been examined through human studies, which demonstrated soy β-conglycinin to reduce iron bioavailability (Cook, Juillerat, Hurrell, Dassenko, & Lynch, 1994). Subsequently, future approaches to enhance iron bioaccessibility may target the improvement of 7S protein digestibility (e.g. through food processing or genetic approaches), in addition to existing strategies to reduce anti-nutritional compounds and enhancing iron content.

6.5. Conclusion

This study highlights the fundamental properties of iron(III)-binding peptides derived from pea protein hydrolysates, which are rich in hydrophilic and polar amino acids, particularly Glu. In corroboration, analysis by FTIR suggested that the main mode of Fe-complexation was *via* carboxylate. Functional classification of the IMAC isolated fraction showed that most iron-binding peptides were from the vicilin fraction, suggesting the importance of vicilin digestibility in relation to iron bioaccessibility from pea protein matrices. Fifteen novel peptides were identified through bioinformatics as potential

candidates for iron bioaccessibility enhancement. These peptides may be further examined as isolated carriers of iron(III) for oral supplementation or food applications, or generated *in situ* during digestion. In the case of isolated peptides, studies are required to optimize the iron-to-peptide ratio and extent of peptide purity necessary for enhancing iron bioaccessibility under physiological conditions.

Chapter 7. Effects of pea protein hydrolysates on the function of gut microbial communities during iron fortification *in vitro*

The previous chapters of this thesis have supported that pea protein hydrolysates can enhance the small intestinal bioaccessibility of fortified ferric iron salt, likely through the iron-binding properties of its constituent soluble peptides. However, it is evident that a considerable proportion of the fortified iron remains insoluble following small intestinal digestion. This fraction is delivered to the lower gut along with undigested proteins, both of which become substrates for colonic fermentation. Whilst colonic iron absorption is feasible, the accrual of accessible colon iron can render the production of reactive oxygen species (ROS) and lead to dysbiosis (Rusu et al., 2020). On the other hand, nascent research *in vitro* has suggested that glycated pea proteins may possess prebiotic effects (Świątecka, Narbad, Ridgway, & Kostyra, 2011), which can offer potential remediation of some undesirable effects of gut iron.

To examine the effects of co-administering different pea proteins (intact protein and hydrolysate) with the fortified iron mineral salts on markers of the human gut microbiota, this chapter will study iron bioaccessibility and gut fermentation products after sequential simulated gastrointestinal digestion and colonic fermentation *in vitro*. Similar to previous studies, iron bioaccessibility is examined using solubility as a proxy at each of the digestion sites. As small intestinal digestion states (fasted versus fed) was not of key interest in this study, the INFOGEST *in vitro* gastrointestinal model (Egger et al., 2016) was selected, with

adaptions made to include colonic fermentation using fresh human faecal cultures. Following 24 hours of batch fermentation, the effects on the gut microbiome were subjected to 16sRNA analysis for microbial sequencing, and short-chain fatty acids (SCFAs)as postbiotic products. The relevant ethical requirements for the use of human faecal cultures have been approved and updated for the year (ID: 2056152).

This chapter contains the fourth research study as published in *Food & Function*. The version included has been peer-reviewed without editorial copyediting, typesetting and proofreading, with the reference style adapted to match that of the thesis. The full reference can be found in the preface.

Hydrolysis of pea protein differentially modulates its effect on iron bioaccessibility, sulfur availability, composition and activity of gut microbial communities *in vitro*

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ABSTRACT:

Both plant proteins and iron supplements demonstrate high susceptibility to escape gastrointestinal digestion and absorption, hence are often present throughout colonic fermentation. Whilst colonic iron delivery may adversely affect the gut microbiota and epithelial integrity, nascent evidence suggests that pea proteins may possess beneficial prebiotic and antioxidant effects during gut fermentation. This study investigated the interaction between exogenously added iron and pea protein isolate (PPI) or pea protein hydrolysate (PPH) during *in vitro* gastrointestinal digestion and colonic fermentation. Results revealed that enzymatic hydrolysis mitigated the crude protein's inhibitory effects on iron solubility during small intestinal digestion. Colonic fermentation of iron-containing treatments led to an increase in iron bioaccessibility and was characterized by a loss of within-species diversity, a marked increase in members of *Proteobacteria*, and eradication of some species of *Lactobacillaceae*. Although these patterns were also observed with pea proteins, the extent of the effects differed. Only PPI displayed significantly higher levels of total SCFAs in the presence of iron, accompanied by greater abundance of *Propionibacteriaceae* relative to other treatments. Additionally, we provide evidence that the iron-induced changes in the gut microbiome may be associated with its effect on endogenous sulfur solubility. These findings highlight the potential trade-off between protein-induced enhancements in fortified iron bioaccessibility and effects on the gut microbiome, and the role of iron in facilitating colonic sulfur delivery.

7.1. Introduction

Iron is an essential trace element with a central role in most biological systems, notably as an enzymatic cofactor in electron transfer and catalysis. However, the poor aqueous solubility of its thermodynamically favoured form [Fe(III)] (10^{-18}M, pH 7.0) makes iron absorption a challenge in the human diet (Ems, St Lucia, & Huecker, 2017). Specifically, Fe(III) is highly polar and prefers O-donor ligands, which facilitates rapid hydrolysis into insoluble oxides and hydroxides upon dissolution in fluids(Sánchez, Sabio, Gálvez, Capdevila, & Dominguez‐Vera, 2017). Upon the ingestion of dietary iron, this leads to precipitation inside the oxygen-rich environment of the human small intestine, its major absorption site. In the absence of lumenal soluble ligands for the dietary iron, only a limited fraction of the free iron becomes bioaccessible, that is, maintained in a soluble form available for absorption into the body (Etcheverry, Grusak, & Fleige, 2012).

Amongst factors related to host biology and/or inadequate intake, poor dietary iron bioaccessibility is a prevailing contributor to iron deficiency, the most prevalent micronutrient disorder worldwide (Bailey, West, & Black, 2015). Iron deficiency and the associated anaemia has long-ranging effects on health, particularly towards energy metabolism, immunity, and productivity. The condition affects approximately 40% of the population in developing countries, whom largely subsist on plant-based diets containing non-heme iron, mostly as Fe(III) (Rusu et al., 2020). Despite oral supplementation strategies being developed to combat iron deficiency, non-heme iron bioaccessibility continues to be impeded by iron's tendency to interact with plant matrix components

rendering it insoluble. Examples include plant proteins, phytate, and polyphenols (Ems et al., 2017).

Limited small intestinal iron absorption due to poor bioaccessibility or impaired mucosal uptake (e.g. during elevated immunity (Bartosik et al., 2022)) increases lower gut iron delivery. Colonic iron absorption is feasible but less efficient (~14% of duodenal efficacy (Yilmaz & Li, 2018b)), largely attributed to the presence of the gut microbiota that limits iron uptake by the host through both competition and regulatory mechanisms(Das et al., 2020). An increase in intraluminal iron is linked to excessive generation of reactive oxygen species (ROS) due to the redox activity of iron in the Fenton reaction. The production of ROS give rise to a cascade of events associated with acute colonic inflammation, including cytotoxic damage from lipid, protein and DNA oxidation, and reduced systemic iron absorption from hepcidin upregulation (Koskenkorva-Frank, Weiss, Koppenol, & Burckhardt, 2013). Studies have also consistently connected increased lower gut iron supply with the replication and virulence of colonic pathogens, as well as altered short-chain fatty acid (SCFA) production patterns (Yilmaz & Li, 2018b). The quantity and ratio of gut-mediated SCFAs such as acetate, propionate and butyrate play critical roles in health; including anti-inflammatory properties, maintaining intestinal cell integrity and enhancing iron absorption (Soriano-Lerma et al., 2022). This implores a need to examine SCFAs as markers of gut health during iron supplementation, where existing evidence has been mixed given variations in host state *in vivo* (e.g. during anaemia) and the iron form/dosage used (Puga et al., 2022).

Iron fortification *via* food matrices have lower bioavailability than iron oral supplements, but are also deemed safer with lower toxicity (Bloor, Schutte, & Hobson, 2021). We previously found that enzymatically produced pea protein hydrolysates (PPH) enhanced the *in vitro* small intestinal bioaccessibility of fortified Fe(III), a finding likely attributable to the release of soluble iron-binding peptides during *in vitro* digestion (Zhang, Stockmann, Ng, & Ajlouni, 2022). Subsequent characterization of the Fe(III)-binding soluble fraction revealed that the sulfur-containing amino acids Cys and Met were notably absent (Zhang et al., 2023). This was consistent with the literature that Cys and Met are generally concentrated in low-digestible albumins or insoluble residue in peas (Rubio et al., 2014), which posits their likelihood for colonic delivery as fermentation substrates or targets of ROS oxidation (Hoshi & Heinemann, 2001). Given some recent preliminary evidence suggesting the antioxidant and prebiotic effects of pea proteins within the gut (Wang et al., 2022), the current investigation aims to investigate the possible prebiotic function of PPI and PPH when fortified with iron. This includes the modulatory effects of these pea protein fractions on iron and sulfur bioaccessibility dynamics throughout digestion, as well as on the biomarkers of gut health (SCFA and gut bacterial composition) during *in vitro* colonic fermentation using human faecal material as the microbiota source.

7.2. Materials & Methods

7.2.1. General reagents and equipment

Common chemicals and consumables used in the study were at reagent grade and purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Pea protein isolate (PPI) from golden field peas was acquired from Pure-Product Australia (Mascot, New South Wales, Australia). Protease M 'Amano' (peptidase from *Aspergillus oryzae*, activity 10,000 U/g) was kindly provided by Wilmar Bioethanol (North Sydney, New South Wales, Australia). Wheat phytase (6-phytase, activity 50 U/g), α-Amylase (from *Aspergillus oryzae*, activity 150 U/mg) and pepsin (from pig gastric mucosa, activity 2500 U/mg) were purchased from Sigma-Aldrich. Pancreatin was acquired from Thermo-Fisher Scientific (Scoresby, Victoria, Australia). Short-Chain Fatty acid standard mixture (#28679) was acquired from Cayman chemical (Ann Arbor, Michigan, USA). All experiments were performed in triplicate, with reagents prepared using Milli-Q water (≤18 Ω) produced in-house using a Synergy UV Millipore System (Merck Life Sciences, Bayswater, Victoria, Australia).

7.2.2. Enzymatic hydrolysis of pea protein isolate (PPI)

PPI was hydrolyzed with the enzymes phytase and protease M as previously described(Zhang et al., 2022). In summary, a 10% (w/w) PPI dispersion containing phytase at 0.8 U/g of the PPI solids was incubated for 3 hours at 50°C (ZWYR-240, Labwit Scientific, Shanghai, China), with the pH maintained at 5.0 every hour using 1 M HCl/NaOH. Protease M was then added to the mixture at 400 U/g of PPI solids and was maintained under the same incubation conditions for an additional 3 hours, after which the

enzymes were inactivated by bringing the pH to 10 using 1 M NaOH. The PPH were recovered by adjusting the pH back to 5.0, prior to centrifugation at 2000 *x g* for 15 min (Allegra X-12R, Beckman Coulter, Land Cove West, New South Wales, Australia). The pellets were frozen at −20°C and lyophilized.

7.2.3. Total Nitrogen analysis

The protein contents of the PPI and PPH powders, as well as the *in vitro* digested fractions undergoing colonic fermentation were determined by the Dumas Combustion method (LECO TruMac CN, Castle Hill, Australia) at a furnace temperature of 1250°C. For the PPI and PPH powders, a triplicate of 1 g was used for analysis. The soluble supernatant and their corresponding sediments following *in vitro* small intestinal digestion were analyzed wet as individual sample replicates. All results were interpreted using a Nitrogen conversion factor of 5.51 for legumes (Fujihara, Sasaki, & Sugahara, 2010).

7.2.4. Simulated gastrointestinal digestion

7.2.4.1. Preparation of soluble Fe(III)-peptide mixtures

Pea protein suspensions with or without iron were prepared as previously reported (Zhang et al., 2022). PPH and PPI were each dissolved in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (0.1 M, pH 7.0) to a final concentration of 1% (w/v). $FeCl_3$ stock solutions were separately prepared at 15 mM. For each sample, 5 mL of the $FeCl_3$ solution (equiv. 4.19 elemental iron) was added to 10 mL of the 1% PPH or PPI, or control solutions of 0.1 M HEPES without proteins by stirring for 20 min at RT. The final mixtures (15 mL each) were subjected to the *in vitro* digestions.

7.2.4.2. Simulated oral, gastric and small intestinal digestion

The harmonized static INFOGEST model was adapted with simulated oral, gastric and intestinal fluids prepared per original (Minekus et al., 2014). The same incubator for enzymatic hydrolysis was used for each of the digestion stages at 37°C. For the oral phase, 5 mL of simulated saliva (containing 4 mL electrolyte oral stock solution at 1.25x concentration, 1.5 mM $CaCl_2$ and 150 U mL^{-1} α-amylase, pH 7.0) was added to each sample, and incubated for 2 min. Gastric digestion was implemented by the addition of 9.1 mL 1.25x gastric stock solution (containing 0.15 mM $CaCl_2$ and 4000 U mL^{-1} pepsin), with 1 M HCl used to adjust pH to 3.0. The samples were incubated for 2 h, prior to terminating gastric digestion by adding 16 mL of 1.25x intestinal stock solution (containing 0.6 mM $CaCl_2$ and 250 U mL^{-1} pancreatin) and 2.5 mL of 200 mM bile, with each sample further adjusted with 1 M NaOH to reach pH 7.0. Small intestinal digestion then took place for 2 h. The intestinal chymes were then centrifuged at 2000 *x g* for 10 minutes at 20°C, with the supernatant removed and contained the bioaccessible fraction and the insoluble sediment immediately continued into colonic fermentation.

7.2.5. Simulated colonic fermentation

7.2.5.1. Faecal culture preparation

A fresh faecal sample from a healthy 26-year-old female was collected to prepare stock cultures within 2 h of defecation. The donor has not received antibiotics or taken probiotics for the previous 12 months. The faecal sample was homogenized in a stomacher bag with filter lining (250 μm), in pre-N_2 flushed 0.1 M phosphate buffered saline (pH 7.0) at 1:5 w/v, with the filtered fluid collected for colonic fermentation. The relevant consent

and requirements for the use of human faecal cultures have been approved by the Human Research Ethics Committee at The University of Melbourne (ID: 2056152).

7.2.5.2. Basal medium preparation

The fermentation medium was prepared as previously described(Zhang et al., 2018), mimicking the chyme of a diversified diet. Briefly, 2.5 g each of potato starch, peptone and tryptone, 2.25 g each of KCl and yeast extract, 4.5 g NaCl, 2 g mucin, 1 g pectin, 1.5 g casein, 0.75 g $NaHCO_3$, 0.4 g L-Cysteine HCl, 0.62 g $MgSO_4 \cdot 7H_2O$, 0.5 g guar gum, 0.25 g KH_2PO_4, 0.2 g bile salts, 0.55 g $CaCl_2$ and 0.5 mL Tween 80 were suspended in 500 mL of deionised water, and autoclaved at 121°C for 40 min (3041 VD, Thermoline Scientific, Wetherill Park, Australia).

7.2.5.3. Batch colonic fermentation and bacterial enumeration

Colonic fermentation and bacterial enumeration procedures were adapted from previous research in our laboratory (Zhang et al., 2018). The insoluble fraction from each replicate following intestinal digestion was mixed with 2 mL of fresh faecal stock culture and 3 mL of basal medium, both prewarmed to 37°C. The samples were placed in anaerobic chambers with a Gaspak (AnaeroGen™, ThermoFisher Scientific, Scoresby, Australia), prior to flushing with N2 gas and tightening the lids. Chambers were placed in an incubator at 37 °C at 2 *x g* for 24 h. Following fermentation, the effluent samples were centrifuged at 2000 *x g* for 10 minutes at 20°C with the soluble fraction stored at –20°C for further analyses. Total plate counts of aerobic and anaerobic bacteria took place prior to, and after 24 h of simulated colonic fermentation using Plate Count Agar in triplicates and a spread

plate technique. The incubation conditions were 37°C for 48 h under aerobic and anaerobic conditions.

7.2.6. Elements analysis

All samples were digested and analyzed under a 5% HNO_3 and H_2O_2 sample matrix using a Perkin Elmer 8300 DV Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (Glen Waverley, Australia) according to our previous work(Zhang et al., 2018). Automatic sample injection (S10) was operated by the Syngistix v3.0 software (Perkin-Elmer, Glen Waverley, Australia). Calibration curves were constructed using multi-element standards (ICP-AM-17 and ICP-AM-12 Solution A, High-purity standards, Charleston, United States). Background correction was applied with multiple emission lines viewed to check for spectral interference. Random and targeted repeat analyses were performed to provide confidence.

7.2.7. Short-Chain Fatty Acids (SCFAs) analyses

7.2.7.1. Sample preparation

The SCFAs content in the supernatant after colonic fermentation was determined by gas chromatography (Agilent gas chromatographer (GC) (7890B, California, USA) coupled to a flame ionization detector (GC-FID). The sample preparation was adapted from a methodology previously optimized for faecal liquids (Scortichini, Boarelli, Silvi, & Fiorini, 2020). Briefly, 250 µL of the supernatant was acidified with 200 µL of 50% sulfuric acid (w/v) and vortexed for 1 min. Previously prepared 4-methylvalerate solution (50 µL, concentration 109.5×10^3 µM) in diethyl ether was then added to achieve a final sample

concentration of 450 µM as an internal standard. The acidified sample was then extracted with 1 mL of diethyl ether and centrifuged for 5 min at 2800 *x g*, with the organic phase transferred into a micro-centrifuge tube. The extraction was repeated three times, with 1.5 mL final volume transferred into GC vials for analysis.

7.2.7.2. Gas Chromatography (GC-FID) analysis

The GC-FID was coupled with an autosampler (7693 Agilent, California, USA) and an autoinjector (G4513A Agilent, California, USA) using the analytical method adapted from Gu et al. (2019). A Nukol capillary column (15 m × 0.53 mm internal diameter with 0.5 µm film thickness, Sigma-Aldrich, Castle Hill, Australia) was used, with helium as the carrier gas at a flow rate of 6 mL/min. The initial oven temperature was 100°C for 0.5 min, ramped up at 12.5°C/min to 180°C and held for 1 min, before increasing again at a rate of 20°C/min to 200°C and holding for 10 min. The FID temperature was set at 240°C with the injection port set at 200°C. Sample injection volume was 2 µL, with a split ratio of 5:1. Standard curves of the analytical standards were prepared by serial dilutions of the SCFAs standard mixture containing acetic, propionic, *iso*-butyric, *n*-butyric, *iso*-valeric and *n*-valeric acids.

7.2.8. Microbial profiling

7.2.8.1. DNA extraction, 16S rRNA gene amplification and sequencing

The baseline culture prior to fermentation, and colonic supernatant of each treatment after fermentation (200 µl per aliquot) were stored in three volumes of DNA/RNA Shield™ solution (Zymo Research, California, USA) as recommended by the manufacturer. Samples were frozen at -20°C and delivered to Australian Genome Research Facility Ltd for DNA extraction and 16S rRNA sequencing.

DNA extraction was performed using the DNeasy® PowerSoil® Pro Kit (Qiagen GmbH, Hilden, Germany). Polymerization was conducted using common primers targeting the V1-V3 region of bacterial 16S rRNA (27F 'AGAGTTTGATCMTGGCTCAG' and 519R 'GWATTACCGCGGCKGCTG'), with a read length of 300bp. The amplicons were sequenced on an Illumina MiSeq (San Diego, California, USA) platform by the MiSeq Control Software (MCS) v3.1.0.13 with Real Time Analysis (RTA) v1.18.54.4 running on the instrument computer. The Illumina DRAGEN BCL Convert 07.021.624.3.10.8 pipeline was used to generate the sequence data.

7.2.8.2. Taxonomic assignment

Taxonomic profiling was performed by QIIME2 as described by Bolyen et al. (2019). The demultiplexed raw reads were primer trimmed and filtered using the cutadapt plugin, followed by denoisation with DADA2 (Callahan et al., 2016) (*via* q2-dada2). Taxonomy was assigned to ASVs using the q2-featureclassifier(Bokulich et al., 2018), and clustered to operational taxonomical units (OTUs).

7.2.9. Data pre-processing and analyses

Bioinformatics and taxonomic visualisations were carried out with the MicrobiomeAnalyst pipeline (Chong, Liu, Zhou, & Xia, 2020) and its companion MicrobiomeAnalystR package (https://github.com/xia-lab/MicrobiomeAnalystR) unless otherwise stated. To reduce noise and low-level contamination for downstream analysis, the sequenced raw count data was filtered using the low count and low variance filters available through the platform, where a combined total of 32 features were removed. The prevalence filter was set to retain values where 10% of each OTU had >5 counts, whereas the variance filter removed features that are close to constant throughout the experiment conditions (cut-off: 10% interquartile range). Subsequent to data filtering, a rarefaction curve was generated to evaluate the adequacy of sampling depth.

The filtered raw count data were normalized using relative log-expression (RLE) for further analyses. Most microbial differential analyses (alpha- and beta-diversity indices, univariate one-way analysis of variance (ANOVA) and Linear discriminant analysis effect size analysis (LEfSe)) were performed through the MicrobiomeAnalyst pipeline, with the false discovery rate (FDR) set at 0.1 based on the Benjamini-Hochberg procedures. Both alpha- and beta-diversity analysis were examined at the feature (OTU) level across treatments.

General statistical analyses were carried out with data imported into Minitab® 20 (Minitab LLC, Pennsylvania, USA). T-tests were performed on the total nitrogen data comparing each pea fraction with their iron fortified counterparts. Differences in

bioaccessible iron and sulfur, SCFAs and total viable count among samples were analyzed with univariate ANOVA, and differences in alpha-diversity indices were analyzed with two-way ANOVA. Fisher's LSD was used as the post-hoc method where applicable. Pairwise Spearman's rank correlation was performed between SCFAs, intestinal and colonic iron and sulfur, and normalized counts of significant bacterial families as identified by ANOVA. All tests for significance were two-sided at 95% confidence, with uncertainty of replicate determinations reported to two significant figures.

7.3. Results

7.3.1. Enzymatic hydrolysis of pea proteins altered the pattern of fortified iron bioaccessibility

This study analyzed the iron found in the soluble supernatant following simulated *in vitro* small intestinal digestion and colonic fermentation as a proxy for bioaccessibility. The iron-fortified pea hydrolysate (PPH + Fe) showed the highest solubility after small intestinal digestion at 0.53 ± 0.052 mg, or 13% of the total 4.19 mg of fortified iron (**Table 19**). The $FeCl_3$ control (Fe) showed comparable levels with 0.39 ± 0.10 mg iron (9.3%) being soluble. However, the addition of fortified iron as $FeCl_3$ to unhydrolyzed PPI (PPI + Fe) led to significantly ($P < 0.05$) lower iron solubility than that of the PPH + Fe, with only 0.093 ± 0.016 mg (2.2%) of the 4.19 mg of iron loaded being soluble. This indicates that the enzymatic hydrolysis of PPI mitigated its inhibitory effects on iron bioaccessibility during small intestinal digestion, and enhanced iron bioaccessibility by 5.7-fold compared to native PPI.

A fraction of the small intestinal insoluble iron in PPI + Fe was released in the colon, where it contributed significantly ($P < 0.05$) to a higher level of soluble Fe than PPH + Fe in the colon from fermentation (PPI + Fe: 0.34 ± 0.082 mg, PPH + Fe: 0.21 ± 0.016 mg, **Table 19**, equivalent to 8.1% and 5.0% of the total Fe loaded). The iron salt treated sample released 0.29 ± 0.0054 mg iron during colonic fermentation, equivalent to 6.9% of the 4.19 mg iron administered. Nonetheless, PPH + Fe exhibited the highest solubility after all digestion stages at 0.74 ± 0.025 mg (17% of Fe loaded), in comparison with the iron salt at 0.68 ± 0.10 mg (16% of Fe loaded). Furthermore, the PPI + Fe showed significantly ($P <$

0.05) lower solubility at 0.43 ± 0.092 mg (10% of Fe loaded). Based on the total Fe present in each treatment prior to *in vitro* digestion, PPH + Fe, PPI + Fe and the $FeCl_3$ control had 3.6, 3.8 and 3.5 mg of iron remaining insoluble after the intestinal digestion process, respectively.

Table 19. Bioaccessibility distribution of iron and sulfur (in mg) from Fe salt control ($FeCl_3$) and pea protein fractions (PPI and PPH) with and without Fe fortification following *in vitro* digestion and colonic fermentation. Bioaccessibility results were expressed as means ± SD (n = 3). Means followed by different superscript letters within each column of each element differ ($P < 0.05$). Numbers in parentheses indicate the mean element concentration present within the sample excluding digestive fluids.

	Intestinal	**Colonic**	**Total bioaccessible (Intestinal + Colonic)**
Iron			
PPI (0.027)	n.d.*	0.020 ± 0.0022^{a}	0.020 ± 0.0022^{a}
PPH (0.068)	n.d.*	0.074 ± 0.025^{a}	0.074 ± 0.025^{a}
PPI + Fe (4.2)	0.093 ± 0.016^{a}	0.34 ± 0.082^{b}	0.43 ± 0.092^{b}
PPH + Fe (4.3)	0.53 ± 0.052^{b}	0.21 ± 0.016^{c}	0.74 ± 0.056^{c}
Fe control (4.2)	0.39 ± 0.10^{b}	0.29 ± 0.0054^{bc}	0.68 ± 0.10^{c}
Sulfur			
PPI (1.8)	42 ± 0.73^{a}	2.8 ± 0.092^{b}	45 ± 0.80^{a}
PPH (1.7)	40 ± 1.10^{a}	6.6 ± 0.18^{a}	47 ± 1.30^{a}
PPI + Fe (1.8)	24 ± 0.49^{b}	2.9 ± 0.12^{b}	27 ± 1.5^{b}
PPH + Fe (1.7)	22 ± 0.57^{b}	2.8 ± 0.11^{b}	25 ± 0.48^{b}
Fe control (0)	25 ± 1.50^{b}	2.6 ± 0.015^{b}	27 ± 0.60^{b}

* n.d. = below detection limit of 0.06 mg/L analyzed liquid

7.3.2. Iron fortification is linked to lower sulfur solubility

Dietary sulfur is mainly derived from thiol amino acids in proteins (Nimni, Han, & Cordoba, 2007). The $FeCl_3$ solution used in this study is technically sulfur-free (ICP-OES detection limit: 0.752 mg/L), which suggests that the sources of sulfur in this study are

from the endogenous proteins present in the digestive fluids and the pea protein fractions. As shown in **Table 19**, the small intestinal solubility of sulfur from the pea protein derivatives were significantly lower in the presence of iron ($P < 0.05$). PPI and PPH contained 42 ± 0.73 and 40 ± 1.10 mg of soluble sulfur respectively, approximately twice of that found in PPI and PPH with iron (PPI + Fe: 24 ± 0.49 mg, PPH + Fe: 22 ± 0.57 mg), after intestinal digestion. The Fe salt control showed similar level of soluble sulfur to samples fortified with iron (25 ± 1.50 mg), suggesting that the addition of $FeCl_3$ reduces sulfur solubility during small intestinal digestion.

Following colonic fermentation, the soluble sulfur (S) did not differ ($P > 0.05$) amongst PPI (2.8 ± 0.092 mg) and PPI + Fe (2.9 ± 0.12 mg, **Table 19**) treatments. However, PPH had 6.60 ± 0.18 mg of soluble sulfur, which was significantly ($P < 0.05$) higher than PPH + Fe (2.75 ± 0.11 mg) and the salt control (2.60 ± 0.015 mg). Overall, sulfur solubility from non-fortified samples (PPI: 45 ± 0.80 mg, PPH: 47 ± 1.30 mg) were significantly ($P < 0.05$) higher than those fortified with iron (PPI + Fe: 27 ± 1.50 mg, PPH + Fe: 25 ± 0.48 mg, Fe: 27 ± 0.60 mg). This was supported by Spearman's pairwise correlation analysis, which showed an inverse association between small intestinal soluble Fe (Int-Fe) with soluble S during both intestinal digestion and colonic fermentation (Colonic S and Int-S, $r=-0.598$, $P < 0.01$, **Figure 18**).

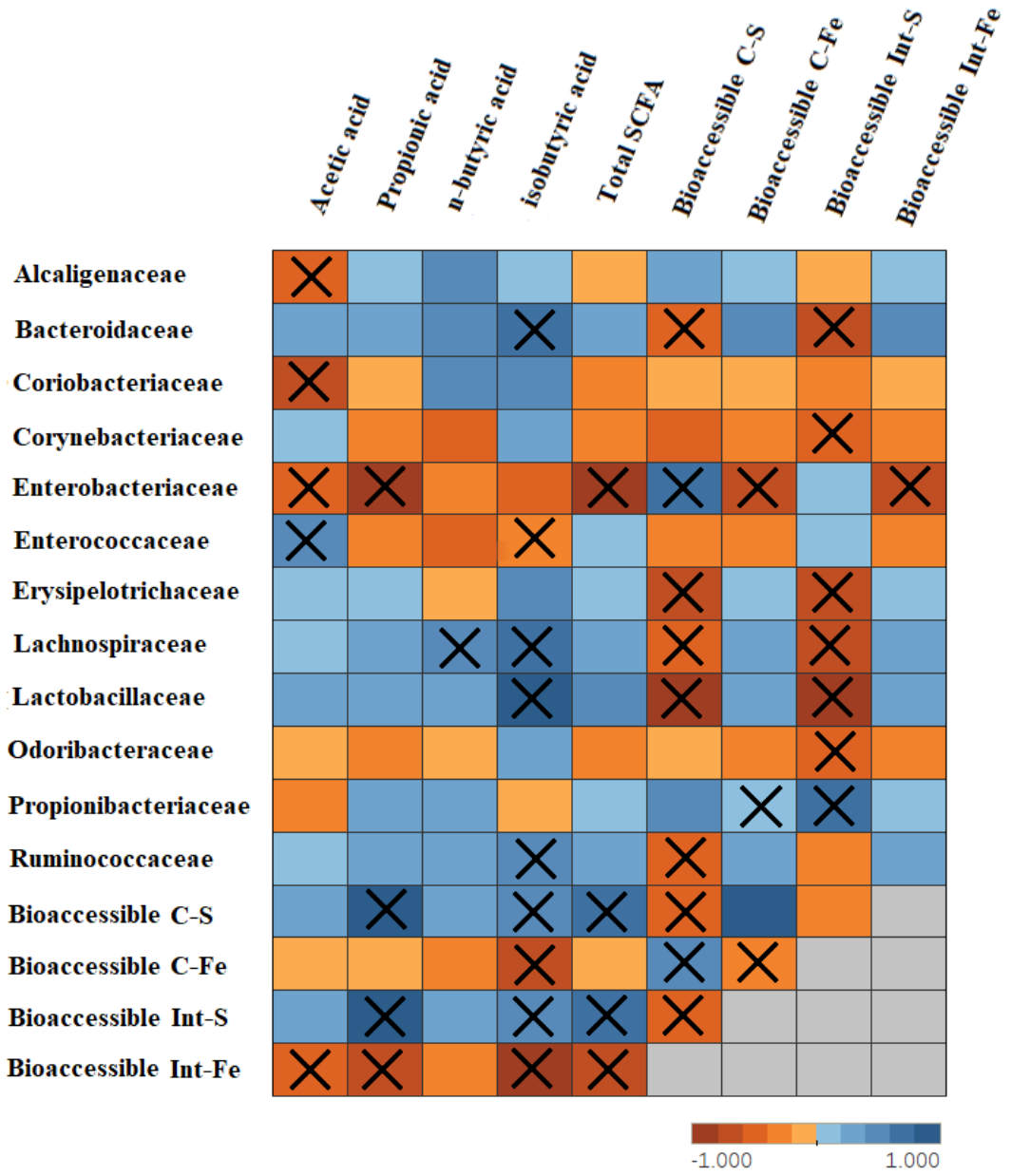

Figure 18. Spearman's pairwise correlation heatmap between normalized abundances of bacterial families and bioaccessible iron and sulfur. C. and Int. respectively represent colonic and intestinal stages. The gradient between red and blue indicates the scale of correlation between negative to positive. Squares with cross (X) indicate statistically significant (FDR-adj $P < 0.05$) values.

7.3.3. Iron fortification increased colonic protein delivery from pea hydrolysates

Total nitrogen analyses showed that PPI contained 70 ± 2.5 mg protein per 100 mg powder, whereas the protein content of PPH was lower at 66 ± 3.3 mg per 100 mg powder. In terms of total protein distribution following small intestinal digestion, there was no statistical difference ($P > 0.05$) between PPI (73% or 51 ± 1.5 mg soluble, 27% or 19 ± 0.54 mg insoluble) and PPI fortified with iron (71% or 50 ± 1.4 mg soluble, 29% or 20 ± 0.96 mg insoluble) out of the 100 mg powder administered. Comparatively, the soluble protein fraction in PPH fortified with Fe (67% or 45 ± 2.2 mg) was significantly ($P < 0.05$) smaller than in PPH alone (78% or 53 ± 2.3 mg). Correspondingly, the insoluble protein fraction in PPH + Fe (33% or 21 ± 1.5 mg) was significantly larger than that PPH alone (22% or 13 ± 1.8 mg). This reveals that the binding of fortified iron to PPH led to a greater fraction of its protein resisted digestion and thus delivered to the gut, which was not observed in PPI.

7.3.4. Changes in gut microbiome patterns and metabolites

Following 24 h of *in vitro* colonic fermentation, samples from each treatment (PPH, PPI, Fe, PPH + Fe, PPI + Fe) were collected for total viable bacterial count, SCFAs analysis and metagenome analysis by 16S rRNA sequencing ($n = 3$ for each treatment). Blank ferments ($n = 3$) were analyzed as quality controls for bacterial count and SCFA production.

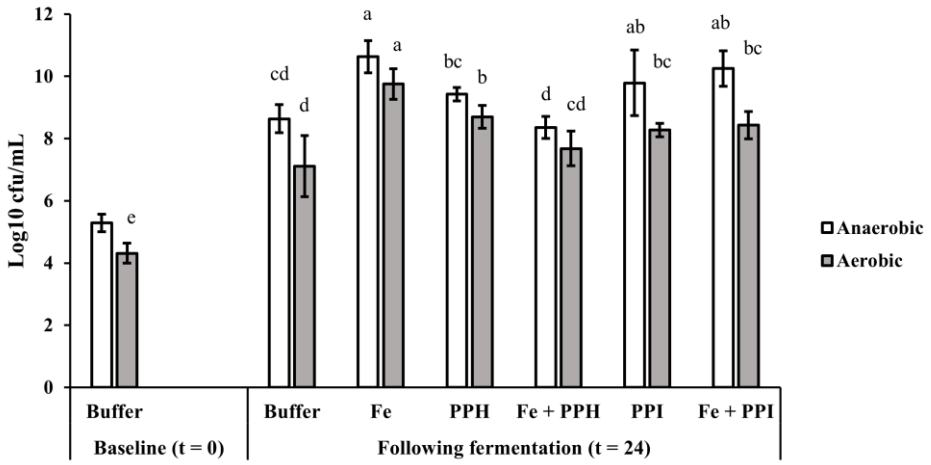

Figure 19. Total anaerobic and aerobic plate counts of the colonic fermented samples before and after 24 h (n = 3). Columns with different superscript letters within each condition (anaerobic or aerobic) are significantly different ($P < 0.05$). Buffer: culture with basal medium, Fe: iron salt control, PPH: pea hydrolysate, PPI: pea protein isolate, Fe + PPH: pea hydrolysate fortified with iron, Fe + PPI: pea isolate fortified with iron.

7.3.4.1. Bacterial abundance and SCFAs

Figure 19 shows the level of anaerobic and aerobic bacteria at 8.6 and 7.1 $\log_{10}$ CFU/mL in control fermentation after 24 h, respectively, up from 5.3 and 4.3 $\log_{10}$ CFU/mL at 0 h. It also shows that the Fe treatment had significantly higher ($P < 0.05$) mean total anaerobic and aerobic bacteria than the control after 24 h of fermentation at 11 and 10 $\log_{10}$ CFU/mL, respectively. Similarly, most treatments containing pea protein fractions had significantly higher levels of total aerobic and anaerobic bacteria relative to the control at 24 h (**Figure 19**).

The SCFAs detected in all treatments after 24 h of fermentation included acetic, propionic and *n*-butyric acids, while *iso*-butyric acid was found in all samples except the Fe only treatment (**Figure 20**). The addition of iron significantly increased the mean total

SCFAs in the pea protein free control (PP-free control: 0.76 mM, Fe: 1.3 mM) (**Fig 20-a**). Iron fortification in the PP-free blank also increased acetic acid production (**Fig 20-b**), but significantly reduced levels of propionic acid (**Fig 20-c**) and *n*-butyric acid (**Fig 20-d**). In contrary, iron fortification significantly reduced total SCFAs in the PPH treatment (**Fig 20-a**) (PPH: 1.9 mM, PPH + Fe: 1.3 mM). However, the total SCFAs in PPI treatment were not affected ($P > 0.05$) by Fe fortification. For pea protein samples without added iron, both PPH and PPI had greater content of total SCFAs as compared to the Fe control treatment (PPI: 1.6 mM, PPH: 1.9 mM, Control: 0.76 mM).

Comparable concentrations of acetic acid were found amongst samples containing pea fractions, although iron addition decreased the acetic acid production in PPH (**Fig 20-b**) (PPH: 1.3 mM, PPH + Fe: 0.87 mM, $P < 0.05$). Iron fortification also significantly ($P < 0.05$) reduced levels of propionic acid in PPH (Fig 3-c) (PPH: 0.42 mM, PPH + Fe: 0.25 mM), but elevated its production in PPI (PPI: 0.30 mM, PPI + Fe: 0.41 mM). Whilst no differences were observed in *n*-butyric acid production amongst samples with pea fractions, PPH fortified with iron showed lower levels of *iso*-butyric acid than all other samples (**Figure 20-d and 20-e**).

7.3.4.2. General trends in bacterial community profile

For microbial profiling, 426 operational taxonomical units (OTUs) were identified according to the Greengenes database after filtering for quality. The rarefaction curve generated displayed a flat plateau, indicating that increasing data volume did not lead to significant changes in the number of OTUs (*Supplementary Data 3*). Six phyla were

observed in this study, with >98% of the sequences from *Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria* and *Chloroflexi*. Although *Firmicutes* was the dominant phyla from the unfermented baseline culture (mean abundance: 45%), *Proteobacteria* was the most abundant in all treatments following 24 h of colonic fermentation (**Figure 21-a**). Fe and PPH + Fe treatments had highest mean relative proportions of *Proteobacteria* (Fe: 57%, PPH + Fe: 58%), with commensurate reductions in *Firmicutes, Actinobacteria, Bacteroidetes and Fusobacteria. Actinobacteria* was highest in PPI, Fe + PPH and Fe + PPI (means: 0.97%, 0.76% and 1.7%, respectively). Highest abundance of *Firmicutes* in the fermented group was found in Fe + PPI (mean: 35%), followed by PPH (mean: 32%). *Bacteroidetes* was the most abundant in PPH (mean: 33%), whereas *Fusobacteria* as only present in Fe-containing samples including Fe, Fe + PPH and Fe + PPI (means: 0.54%, 0.87% and 0.48%, respectively).

The top 20 families and species are depicted in **Figure 21-b and 21-c**, respectively. Over 93% of all OTUs could be classified at the family level at the minimum. The top 10 abundant families present in all treatments were Enterobacteriaceae, *Bacteroidaceae, Veillonellaceae, Enterococcaceae, Porphyromonadaceae, Ruminococcaceae, Rikenellaceae, Bacillaceae, Lachnospiraceae* and *Coriobacteriaceae*. The family *Alcaligenaceae* was found in all treatments except in Fe, while *Lactobacillaceae* was not identified in any treatment involving fortified iron (Fe, Fe + PPH, Fe + PPI). *Propionibacteriaceae* was only present when pea protein substrates are present with iron (Fe + PPH, Fe + PPI), whereas *Erysipelotrichaceae* was detected in all but pea protein substrates with iron (Fe + PPH, Fe + PPI).

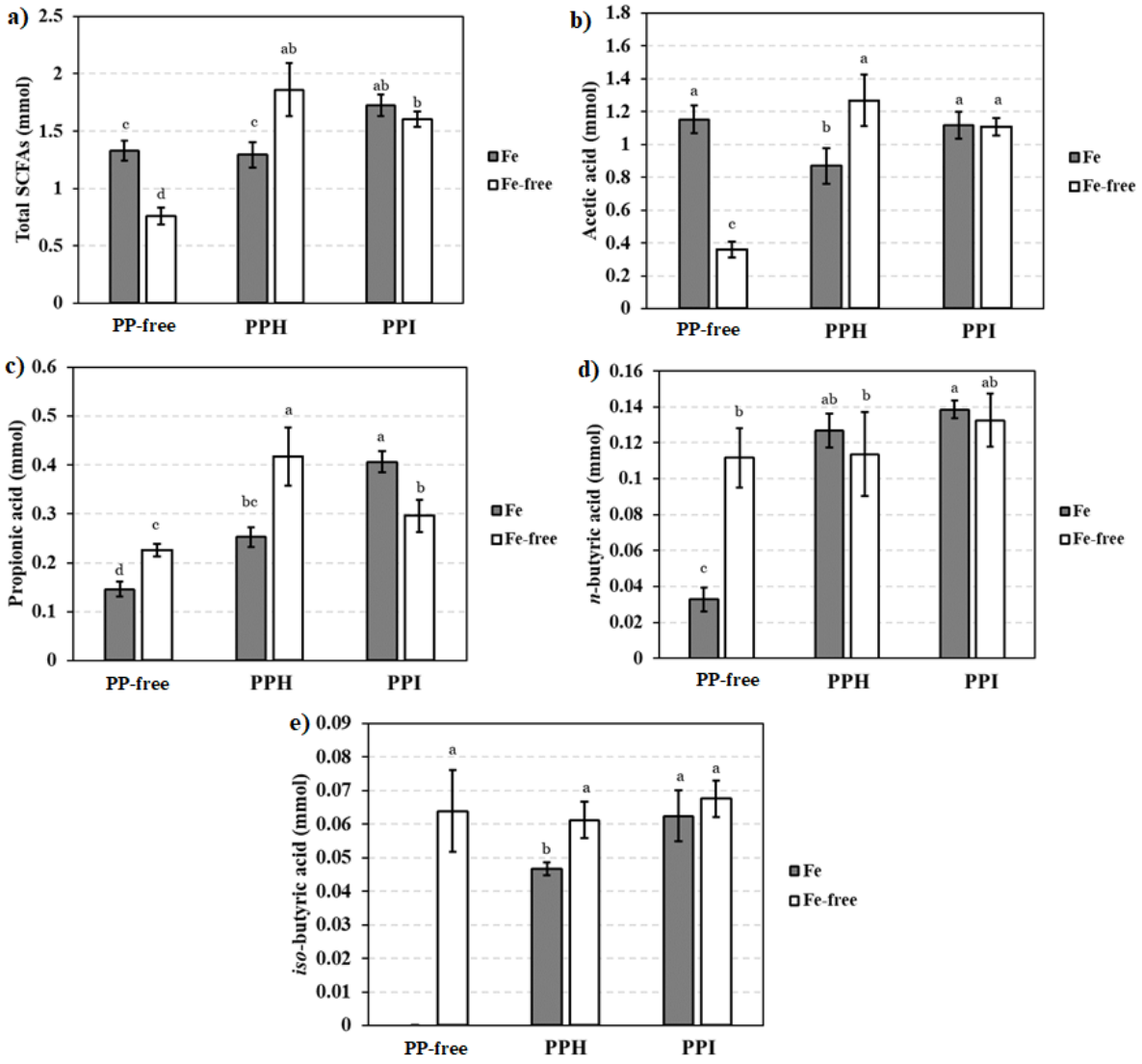

Figure 20. Short-chain fatty acids (SCFAs) produced in each treatment with and without fortified iron (Fe and Fe-free, respectively) after 24 h of colonic fermentation. a) total SCFAs, b) acetic acid, c) propionic acid, d) *n*-butyric acid, and e) *iso*-butyric acid. Values were expressed as means ± SD (n = 3), where treatment groups that do not share a superscript letter are significantly different ($P < 0.05$). PP-free: fermented culture without pea proteins, PPH: pea hydrolysate, PPI: pea protein isolate.

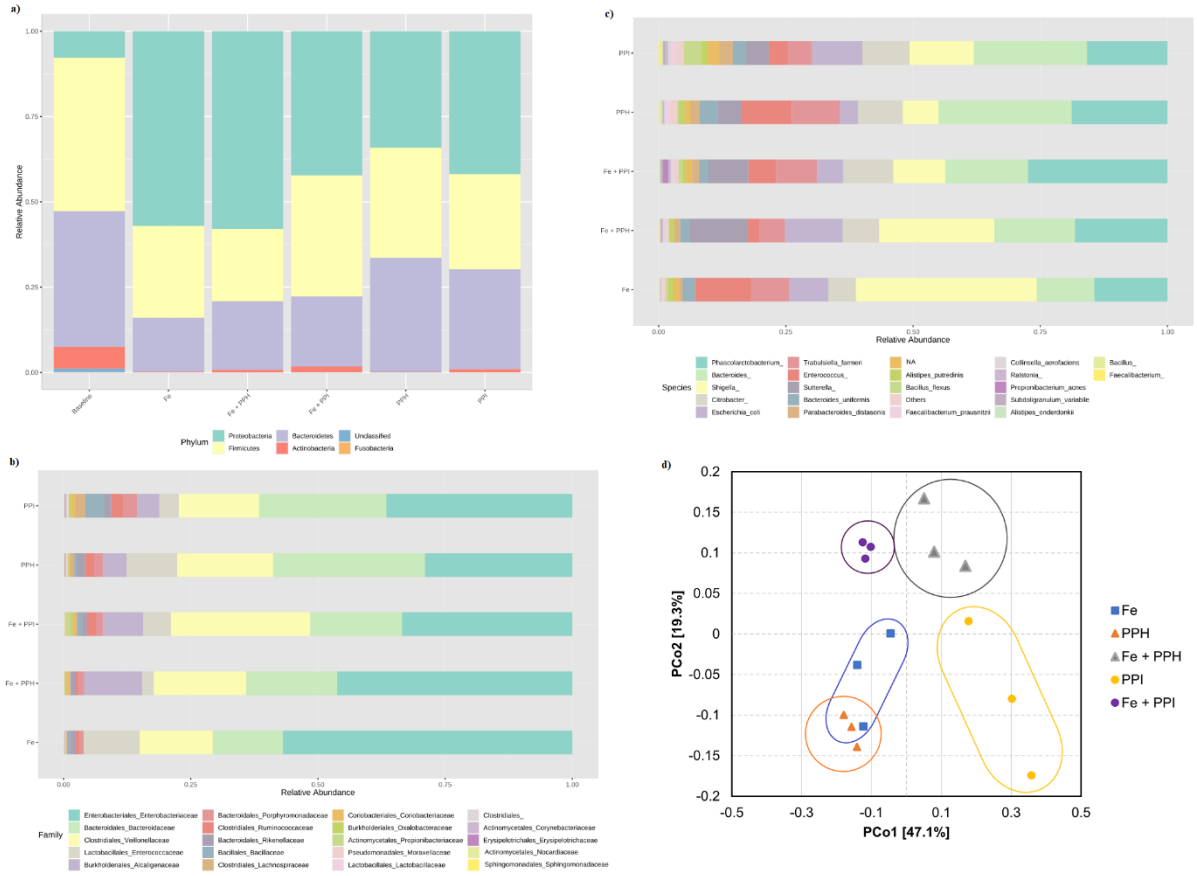

Figure 21. a) Top five phylum-level changes in the gut microbiome of the fermented samples versus the unfermented baseline culture ('Baseline'), with the remainder phyla merged in the 'Unclassified' group. b) and c) displays the top 20 families (prepended by order) and genus (including species where applicable) found in the microbiome of the fermented samples by relative abundance. d) Principal Coordinate Analysis (PCoA) plot as a β-diversity index of the gut microbiome profiles, based on Bray–Curtis dissimilarity between samples at a feature (OTU) level. Each data point represents the microbial community composition of one sample. All values excluding the PCoA plot are merged means of triplicates. Fe: iron salt control, PPH: pea hydrolysate, PPI: pea protein isolate, Fe + PPH: pea hydrolysate fortified with iron, Fe + PPI: pea isolate fortified with iron.

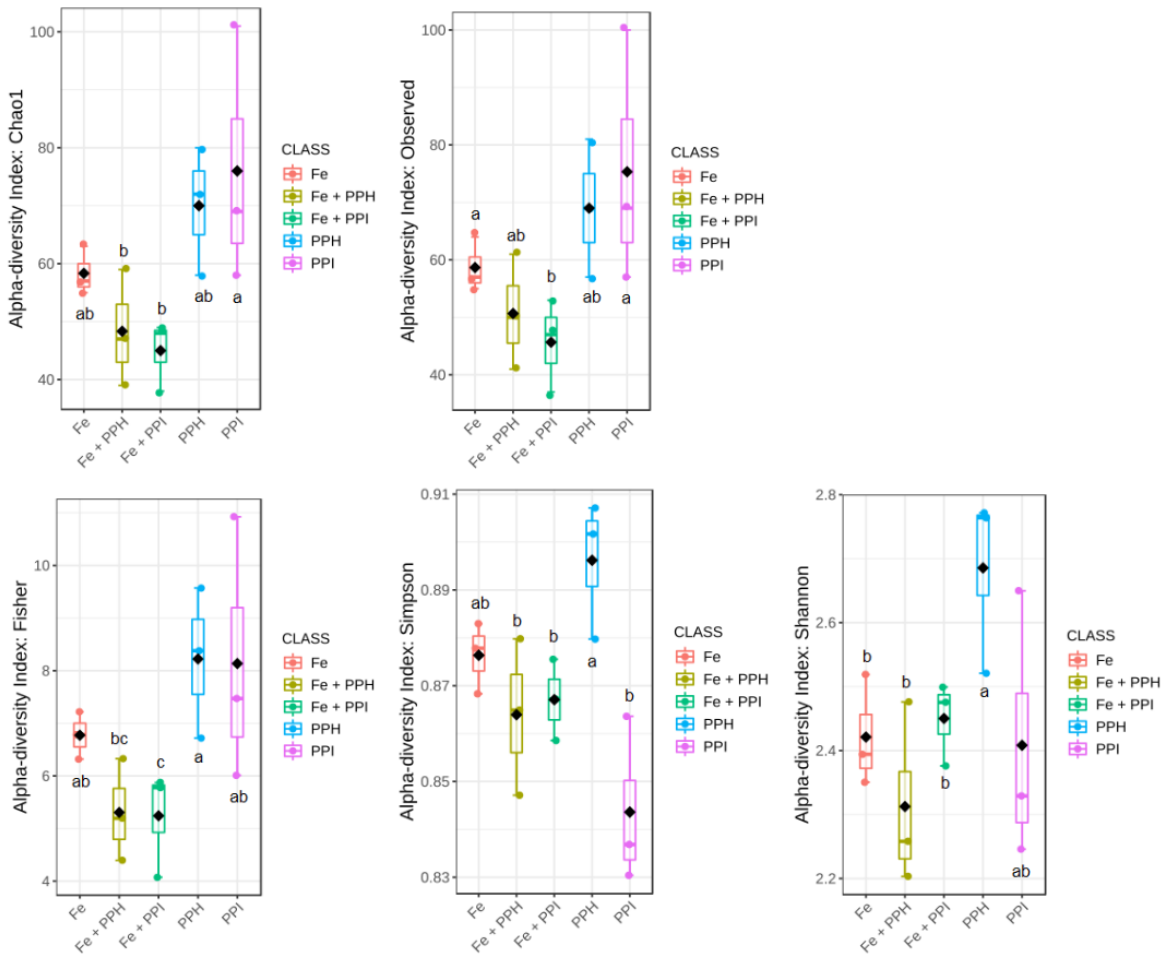

Figure 22. The α-diversity indices of the gut microbiome in the Fe salt control (Fe), pea hydrolysate (PPH), pea protein isolate (PPI), and the latter two groups fortified with iron (Fe + PPH, Fe + PPI). Analyses were conducted at the feature (OTU) level, where treatment groups that do not share a super/subscript letter are significantly different ($P < 0.05$).

7.3.4.3. Richness and diversity analysis

For alpha-diversity analysis, both indices of richness (Chao1, observed species) and diversity (Simpson, Shannon and Fisher's indices) were examined across treatments (**Figure 22**). The degree of similarity between community structures (beta-diversity) was examined through Principal Coordinates Analysis (PCoA), based on the Bray–Curtis

dissimilarity distance. Permutational multivariate analysis of variance (PERMANOVA) and analysis of group similarities (ANOSIM) were performed to evaluate the strength of compositional differences.

As shown in **Figure 21-d**, the amounts of the two maximum variations amongst treatments were explained by the values of the abscissa axis (PCo1, 47.1%) and ordinate axis (PCo2, 19.3%), accounting for 66.4% of total variation. The treatment groups occupied different centroids (PERMANOVA, R-squared: 0.77205, $P = 0.001$), with significantly higher similarity within each group than that between them (ANOSIM, $P < 0.001$).

Results from PCoA analyses revealed that the microbial profiles from PPH and PPI treatments responded differently to iron fortification (**Figure 21-d**). The non-fortified treatments (PPH and PPI) largely lay in the same region in the PCoA plot with negative correlations to PCo2, whilst their iron-containing counterparts have higher PCo2 values. However, whilst PPI shifted to lower PCo1 values in the presence of iron, PPH moved toward higher PCo1 values. These trends are supported by that of alpha-diversity analyses, which showed that the presence of fortified iron significantly ($P < 0.05$) reduced markers of observed species, Fisher index and Chao1 in PPI, with no significant effects on Shannon and Simpson indices. On the other hand, iron addition to PPH led to a significant ($P < 0.05$) reduction in Fisher, Simpson and Shannon indices. Together, these results suggest that whilst iron addition was linked to a general decline in both species abundance and richness when PPI was present, only the species richness was affected in the presence of PPH.

7.3.4.4. Differential abundance analysis

Differential analyses were conducted on the normalized abundances of the five fermented groups (Fe control, PPI, PPH, PPI + Fe, and PPH + Fe) using ANOVA and LEfSe, respectively. Results shown in **Fig. 23** indicated that at the minimum classification level of family, univariate ANOVA revealed that *Propionibacteriaceae* was significantly higher in treatments containing both iron and a pea protein substrate (Fe + PPH and Fe + PPI, $P < 0.01$). This corroborates with findings from LEfSe, which identified *Propionibacteriaceae* as a biomarker for Fe + PPI (adj-$P < 0.01$, LDA score: 2.47). Without fortified iron, PPI is characterized by higher levels of *Bacillaceae* ($P < 0.01$, LDA score: 4.27), *Bacteroidaceae* ($P < 0.05$, LDA score: 3.62), *Porphyromonadaceae* ($P < 0.05$, LDA score: 2.81), Lachnospiraceae ($P < 0.05$, LDA score: 2.75) and Coriobacteriaceae ($P < 0.05$, LDA score: 2.35) from LEfSe. These patterns were confirmed by cluster analyses and ANOVA, although the effects on *Bacteroidaceae* and *Porphyromonadaceae* were no longer significant after adjusting for FDR ($P > 0.05$). *Alcaligenaceae* was identified as a marker for PPH with fortified iron ($P < 0.02$, LDA score: 3.52), where it was notably lower in the iron control without pea protein substrates (ANOVA: $P < 0.001$). Iron treatment alone was characterized by *Enterobacteriaceae* and *Enterococcaceae* from LEfSe ($P < 0.05$, LDA Scores: 4.27 and 3.32, respectively). ANOVA analyses confirmed that *Enterococcaceae* was significantly higher in Fe relative to other treatments, although Fe was not similar in relative abundance to Fe + PPH for *Enterobacteriaceae* ($P < 0.05$, **Figure 23**).

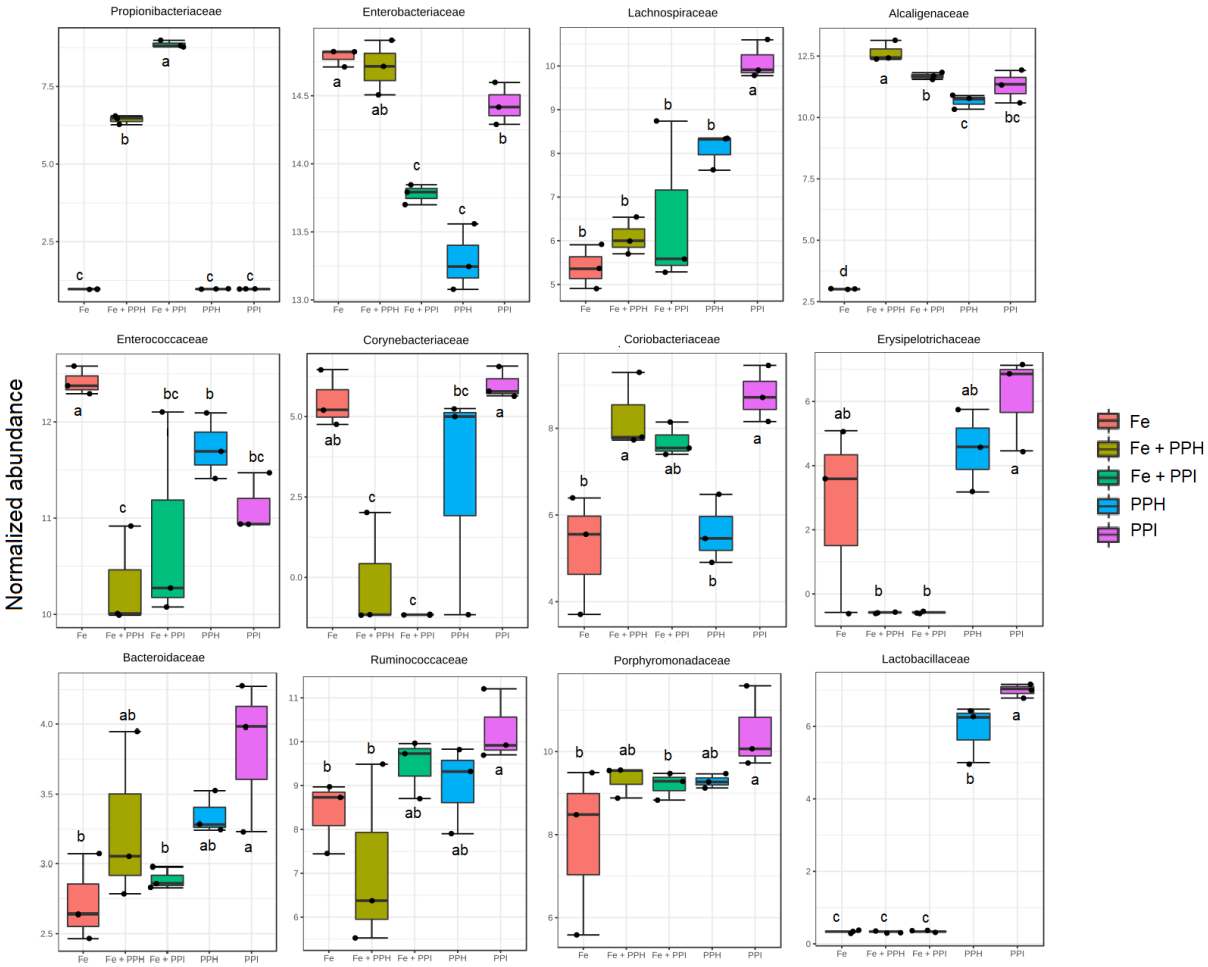

Figure 23. Boxplot of the abundances of 12 significantly different bacterial families as identified from ANOVA, where treatment groups that do not share a super/subscript letter are significantly different ($P < 0.05$). *Bacteroidaceae* and *Porphyromonadaceae* were no longer significant after adjusting for FDR. Fe: iron salt control, PPH: pea hydrolysate, PPI: pea protein isolate, Fe + PPH: pea hydrolysate fortified with iron, Fe + PPI: pea isolate fortified with iron.

7.3.5. Correlation between iron and sulfur availability and SCFAs production and bacterial taxa

7.3.5.1. Elements bioaccessibility and SCFAs

As shown in **Figure 18**, apart from its association to intestinal and colonic bioaccessible sulfur, small intestinal bioaccessible Fe (Int-Fe) was also negatively correlated with the production of all detected SCFAs except *n*-butyric (acetic, r=-0.575, $P < 0.05$; propionic, r=-0.651, *iso*-butyric, r=0.821; total SCFAs, r=-0.71, $P < 0.01$). Similar patterns were observed for Colonic Fe, except the association was only significant for *iso*-butyric (Colonic Fe, r=-0.655, $P < 0.01$). Colonic S and Intestinal S were positively associated ($P < 0.01$) with propionic (r=0.854), isobutyric (r=0.592) and total SCFAs (r=0.693).

7.3.5.2. Gut community abundance and SCFAs

The normalized abundances of several bacterial families, including *Alcaligenaceae* (r=-0.588, $P < 0.05$), *Coriobacteriaceae* (r=-0.636, $P < 0.01$) and *Enterobacteriaceae* (r=-0.593, $P < 0.05$) demonstrated an inverse relationship with the production of acetic acid. *Enterobacteriaceae* was also negatively correlated with propionic acid (r=-0.879, $P < 0.01$), isobutyric acid (r=-0.564, $P < 0.05$) and total SCFAs (r=-0.932, $P < 0.01$). The only family associated *n*-butyric acid production was *Lachnospiraceae* (r=0.457, $P < 0.05$). Several families were positively associated with isobutyric acid with varying strengths, including *Ruminococcaceae* (r=0.566, $P < 0.05$), *Bacteroidaceae* (r=0.677, $P < 0.05$), *Lachnospiraceae* (r=0.707, $P < 0.01$) and *Lactobacillaceae* (r=0.826, $P < 0.01$).

7.3.5.3. Elements bioaccessibility and gut community abundance

Int-Fe was negatively associated with abundances of *Bacteroidaceae* (r=-0.585, P < 0.05), *Erysipelotrichaceae* (r=-0.682, P < 0.01), *Lachnospiraceae* (r=-0.598, P < 0.05), *Lactobacillaceae* (r=-0.843, P < 0.01) and *Ruminococcaceae* (r=-0.598, P < 0.05), but positively associated with *Enterobacteriaceae* (r=0.666, P < 0.01). Similar associations were observed with Colonic Fe for *Bacteroidaceae* (r=-0.682, P < 0.01), *Erysipelotrichaceae* (r=-0.696, P < 0.01), *Lachnospiraceae* (r=-0.732, P < 0.01), and *Lactobacillaceae* (r=-0.886, P < 0.01). Meanwhile, *Propionibacteriaceae* was positively associated with Colonic Fe (r=0.62, P < 0.05). The only family with significant association with sulfur (both Int-S and Colonic S) was *Enterobacteriaceae* (r=-0.736, P < 0.01).

7.4. Discussion

Food protein-derived hydrolysis products are intimately linked to iron bioaccessibility throughout the gastrointestinal tract, one mechanism by which is through increasing iron's solubility required for absorption. Partially hydrolyzed proteins, peptides, and amino acids may enhance intestinal iron bioaccessibility by their ionizable groups forming soluble low MW soluble complexes with iron, thereby circumventing its polynucleation with other counterions (e.g. phosphates, oxides and hydroxides) that decreases iron solublility. Such mechanism is more convoluted in the context of legume proteins, where their affinity for iron is coupled with structures and regions that exhibit hydrolytic resistance against digestive enzymes and brush border amino peptidases (Zannini, Sahin, & Arendt, 2022). Several legume proteins have been shown to inhibit fortified iron solubility, hence bioavailability, *in vivo* (Hurrell et al., 1992; Mayer Labba et

al., 2022), which are consistent with our findings from the native pea protein (PPI). This digestion-resistant fraction containing both legume protein and fortified iron reaches the lower gut, although their combined effects on colonic microbiome markers have hitherto been unexplored until this study.

Enzymatic hydrolysis of the pea protein prior to intestinal digestion mitigated its detrimental effect on small intestinal iron bioaccessibility during iron fortification, with a lower fraction of the soluble iron released within the gut. Such finding confirms that the inverse correlation between iron bioaccessibility and the MW of protein hydrolysates is applicable to pea proteins, given sufficient hydrolysis by both phytase and protease to achieve peptide fragments with sufficient hydrophilicity. During gastric digestion, pea peptic products are formed *in situ* that are known inhibitors of digestive enzymes within the small intestine (Awosika & Aluko, 2019). As such, partial unfolding of the polypeptides (both secondary and tertiary structures) can increase the ionizable groups and iron-binding but restrict its bioaccessibility, due to the poorly soluble complexes formed. A parallel view of both iron bioaccessibility and protein distribution suggests that treatment of PPI by protease and phytase likely enhanced both soluble and insoluble binding iron peptides, which eventuated in improved small intestinal bioaccessibility. Referring to similar static *in vitro* gastrointestinal models employed in previous reports, the 5.7-fold increase in small intestinal iron solubility was consistent with those examining fortified iron with crude versus hydrolysed legume proteins. This includes the 5-fold increase found in our former study with pea hydrolysates (Zhang et al., 2022) and the 3.5- to 6.0-fold enhancement reported in soy hydrolysates by Devaraju et al. (2016). Comparatively, the same pea

hydrolysates that underwent a longer digestion period of 3 h showed a considerably higher increase in iron solubility (Zhang et al., 2022). These results reiterate the critical role of adequate legume protein digestibility in mediating iron bioaccessibility, which in *in vitro* studies may be a function of the digestion protocol conditions employed.

$FeCl_3$ supplementation alone led to increased soluble colonic iron, which promoted conditions associated with an imbalanced microbiome and shifts in SCFA production profile. Despite the low dose of iron used in this study (4.2 mg elemental iron versus 35-65 mg/dose for treating iron deficiency anemia(Bloor et al., 2021)), gut bioaccessibility for all fortified treatments exceeded the 0.4 mmol reported for conventionally accessible intraluminal iron (Yilmaz & Li, 2018b). Consistent with findings by Dostal et al. (2014) in a rodents, iron salt addition led to greater total SCFAs production in colon as a likely result of enhanced bacteria metabolic activity. However, we found that such increase in total SCFAs resulted in altered ratios over favouring acetate (86% of total SCFAs in Fe versus 47% in the fermented treatments without Fe), which was also observed by Poveda, Pereira, Lewis, and Walton (2020). This may be suggestive of selective enrichment in acetogenic species under iron fortification *in vitro*, which is supported by our findings of significant higher total aerobic bacteria count in the presence of Fe. Total aerobic count can indicate an increase in facultative anaerobes such as *Enterococcaceae* and *Enterobacteriaceae*, which are known acetate producers (Macfarlane & Macfarlane, 2012) and identified as LEfSe biomarkers for Fe in this study. Proliferation of the latter is associated with the depletion of butyrate-producing species (Ceccarani et al., 2020), which hinders the ability of acetate to be utilized towards butyrate production *via* cross-feeders. Such shift affects the availability

of butyrate as the source providing 70-80% of total energy for colonocytes (Gasaly, Hermoso, & Gotteland, 2021), as well as a contemporaneous decline in commensal/beneficial species. At the phylum level, we observed an expansion in members of *Proteobacteria* that were most prominent in Fe amongst all treatments. The proliferation of *Proteobacteria* (particularly *Enterobacteriaceae*) has been one recurrent outcome of recent iron supplementation studies (Yilmaz & Li, 2018b). Many pathogenic and/or virulent species of *Proteobacteria* possess a growth advantage under increased iron availability, which can ultimately attenuate host colonic iron uptake *in vivo* through competition (Yilmaz & Li, 2018b).

Reflecting the different composition of the digestion products, we found that iron fortification of the two pea structures led to disparate effects on the gut microbiota. Iron fortification with PPH led to decreased production of the proteolytic metabolite *iso*-butyric acid when compared to PPH alone. However, the addition of iron also significantly decreased ($P < 0.05$) the total SCFA production and species richness found in PPH. Conversely, fortification of Fe with PPI led to a decline in both species' abundance and richness. Consistent with a significant increase in *Propionibacteriaceae*, iron addition to PPI also led to higher levels of propionic acid, where the resultant total SCFAs were the highest observed in all treatments. Despite the baseline differences between PPI and PPH, iron fortification of either fraction led to lower gut microbial diversity compared to without. This may be linked to the loss of potentially important taxa observed in the presence of iron, such as *Lactobacillaceae*, which plays a pivotal role in regulating iron absorption *via*

the production of microbial metabolites *in vivo* (Das et al., 2020). Nonetheless, the higher SCFAs found in presence of pea fractions may partially ameliorate the effects of fortified iron. This is as SCFAs have been proposed as mediators of colonic iron absorption (Soriano-Lerma et al., 2022), with low faecal concentrations observed under iron-deficient conditions in both rodents (Dostal et al., 2012), and in *vitro* (Dostal et al., 2015). Elevated SCFAs production has been one of the only consistent findings regarding the effects of pea protein on the gut to date, amongst an increase in the family *Ruminococcaceae* (Wang et al., 2022*)*. The latter was also observed in our study, where PPI-containing treatments contained the highest relative abundance. *Ruminococcaceae* are associated with butyrate production amongst the other biomarker families of PPI, including *Lachnospiraceae, Coriobacteriaceae* and *Porphyromonadaceae* (Oliphant & Allen-Vercoe, 2019).

Our findings shed lights on the prospective role of sulfur in the modulation of iron bioaccessibility from mineral salts and its subsequent effects on the gut microbiome. Analogous to *in vivo*, sulfur in this study was derived from proteins originating both endogenously (digestive fluids) and from dietary sources (pea proteins). We observed an inverse relationship between bioaccessible iron and sulfur following small intestinal digestion, where iron fortification of samples consistently decreased soluble sulfur regardless of the substrate. Although sulfur is known to be liberated from amino acids (Blachier, Mariotti, Huneau, & Tomé, 2007), the successive increase in colonic soluble sulfur in iron-fortified treatments was not followed by higher levels of soluble sulfur following fermentation. This suggests that fortified iron led to insoluble sulfur-containing fractions following intestinal digestion, which remained intact during colonic fermentation.

It is also possible that sulfur might be released as volatile compounds under such condition. Sulfur-rich food proteins have long been implicated in enhancement of iron bioaccessibility, owing to the ability of low MW digestion products with sulfhydryl groups to reduce Fe(III) to Fe(II) that can form more soluble iron complexes (Horimoto, Tan, & Lim, 2019). However, the high affinity of Fe(III) towards sulfhydryl and carboxyl groups can take precedence over its binding to hydroxyl groups, forming iron chelates with low solubility. Less soluble iron-sulfur chelates have been recognized in polysaccharides in reducing iron bioaccessibility (Wang et al., 2019), although there has been less discussion on the context of proteins and their effects on the gut. Nascent evidence suggested that ferrous sulfate demonstrated mixed effects on rodent gut models relative to other forms of iron supplementation (Seyoum, Baye, & Humblot, 2021), and it remains unclear as to what extent any gut alterations may be attributed to sulfur. The ramifications of indigestible iron-sulfur complexes is worthwhile exploring, with implications for using ferrous sulfate as the gold standard for iron oral supplementation (Bloor et al., 2021), as well as protein-rich sources of iron as the main source of dietary sulfur for most individuals (Carbonero, Benefiel, Alizadeh-Ghamsari, & Gaskins, 2012).

The significant association between bioaccessible gut sulfur and *Enterobacteria* further suggests that protein-derived sulfur may be an intermediate exacerbating the effects of iron fortification during colonic fermentation. Pathogens such as *Escherichia coli* and *Pseudomonas aeruginosa* have been identified to encode genes involved in the production of hydrogen sulfide (H_2S) *via* cysteine degradation (Shatalin, Shatalina, Mironov, & Nudler, 2011), a process that can be further stimulated by dietary sulfur (Carbonero et al.,

2012). Elevated H_2S production has been implicated in the pro-inflammatory effects of high-protein diets, as well as toxic to lactic acid bacteria (Dordević, Jančíková, Vítězová, & Kushkevych, 2021). As such, iron-induced gut sulfur delivery may generate independent effects associated with iron (e.g. an increase in *Enterobacteria*), but also provide additional substrate for increased H_2S that possibly contribute to the obliteration of *Lactobacillaceae*. Whilst H_2S was not specifically measured in this study, we found significantly higher levels of bioaccessible gut sulfur from PPH treatment relative to PPI. This is supportive of our hypothesis that the sulfur-containing proteins from PPH are delivered to the colon with the undigested fraction, where sulfur would have been released during fermentation without the presence of fortified iron forming insoluble complexes.

The present study sheds light on the complex interrelationship between plant protein structure, digestibility, fortified iron bioaccessibility and their association with changes in the gut microbiome. Our findings were limited by the *in vitro* nature that merely reflect acute changes within an adult microbiome, which can be more stable overtime (Teichmann & Cockburn, 2021). Additionally, our analysis of the soluble faecal medium likely excluded some microbial load within the insoluble pellet that may explain the loss of some species. Nonetheless, this investigation highlights a prospective role of fortified iron to increase lower gut sulfur supply, which may be one fundamental mechanism exacerbating iron's deleterious effects on the colonic microbiota. Future studies are urged to examine the robustness of the changes in gut microbiome profile that stem from fortifying iron through pea protein matrices, amidst interindividual differences. The range of proteolytic

metabolites produced should also be explored to elucidate pea proteins' putatively protective effects on the gut, particularly H_2S as a sulfur by-product.

Chapter 8. General Discussion and Conclusion

8.1. Summary and Significance of the Study

Legume seeds are affordable and accessible staple sources of dietary nutrients for populations worldwide. Although the seed matrix itself is known to possess factors that synergistically inhibit elemental bioaccessibility, they are regularly consumed among interventions targeting Fe and Zn deficiency. This study explored the feasibility of legume protein fractions as effective carriers for fortified iron and zinc using *in vitro* gastrointestinal models. The initial studies in this investigation substantiated the propensity of legume proteins to interact with fortified Fe and Zn. Following a series of bioprocessing approaches targeting protein as a bioaccessibility modulator, the peptides responsible for increased Fe solubility were further characterised. Lastly, the effects of the insoluble Fe-containing fractions on the human colonic microbiota were examined.

Whilst phytate content alone has been recognized as the most potent inhibitor of both iron and zinc bioavailability (Sandberg, 2002), results from this research amplifies existing evidence that collegial acts between legume seed proteins and phytate are likely the critical contributors to poor elemental bioaccessibility. Phytate is located in the protein bodies of legume seeds, where the protein-bound fraction is insoluble and refractory to peptic digestion (Dersjant-Li, Awati, Schulze, & Partridge, 2015). The remaining are partially proteolysed during gastric digestion, forming products that are easily aggregated by both phytate and fortified elements under small intestinal digestion conditions, as evidenced in Chapter 4. These proteolytic products can be difficult to digest due to the collective presence of phytate and high ionic strength from fortification and endogenous salts from

digestion fluids, which act as strong kosmotropes that increase hydrophobicity by breaking H-bonds, thereby restricting access to proteolytic enzymes (Selle, Cowieson, Cowieson, & Ravindran, 2012). Moreover, phytate is known to form insoluble ternary complexes with legume protein *via* a cationic bridge (e.g. with Fe, Zn, Ca) under small intestinal pH, where most seed proteins are negatively charged (Zhang et al., 2020). These conditions may catalyse the precipitation of large MW protein aggregates unless the seed protein has been extensively hydrolysed. As such, phytase and protease hydrolysis of the pea protein in Chapter 5 led to a drastic enhancement in pea protein solubility following simulated digestion, corresponding to the increase in fortified Fe and Zn solubility. Here, phytate reduction is stressed not merely for lessening its chelation property, but for its ability to enhance protein digestibility by attenuating its kosmotropic effects. This further enhances the release of soluble peptides that desorb elements from precipitates, and/or form of more soluble element complexes potentially enhancing their bioaccessibility.

Previous investigations have generally attributed phytase-mediated improvements on iron/zinc bioavailability in plant-based foods to phytate hydrolysis, where lower myo-inositol phosphates possess diminished element chelating properties compared to IP6 (Bohn et al., 2008). Coevally, food processing methods employing phytase (e.g. fermentation, germination, enzymatic hydrolysis) can induce a host of microstructural and chemical changes critically linked to protein solubility (Sá, Moreno, & Carciofi, 2019). The subsequent degree of improvement in protein digestibility, which is an interplay between the processing conditions and the initial protein source, may then act as a determinant of elemental bioaccessibility beyond the scope of phytate alone. Differences in protein

digestibility may be partially explanatory of some discrepancies in the effects of food processing on elemental bioaccessibility observed among various studies. For example, several authors have reported no benefits on Fe and Zn bioaccessibility with legume seed germination or fermentation, despite evidence of phytate reduction (Hemalatha et al., 2007; Luo & Xie, 2013). Contrarily, pork and fish proteins have consistently demonstrated ability to enhance non-heme Fe bioavailability, even in phytate-rich meals (Baech et al., 2003). These patterns were corroborated with the findings in Chapter 5, where enhancements in Fe and Zn bioaccessibility were only detected when there were corresponding increases in soluble protein reflecting digestibility. This was commonly observed under the fed intestinal state, where the longer digestion time and higher bile concentration contributed to increased solubilisation of the proteins.

Findings from this thesis further revealed some potential mechanistic relationships between legume protein matrices and the decrease in iron bioavailability. When the soluble Fe(III)-binding peptides from pea protein were separated and characterised in Chapter 6, most of the peptides identified were either low in relative abundance, or did not possess ideal physicochemical properties (e.g. size, hydrophilicity and charge) as ligands for enhancing Fe solubility within the small intestine. The identification of 7S globulins as the major parent source of these Fe-binding peptides suggests that this fraction can inhibit Fe bioaccessibility if undegraded, which is likely as 7S globulins are known to interact strongly with phytate (Hídvégi & Lásztity, 2002), and are poorly digested (Ohanenye et al., 2022). Additionally, the recognition that food processing may produce divergent effects on Fe and Zn bioaccessibility through legume seed proteins (Chapter 5) underscores another

barrier. It is presently acknowledged in the literature that Zn exerts a strong positive impact on both Fe absorption processes and Fe status, where major transporters for both elements are receptive to cellular Zn concentrations (Knez, Graham, Welch, & Stangoulis, 2017). This prompts that interventions targeted solely at Fe may be muted in the absence of adequate Zn status, especially when efforts to enhance dietary Fe bioaccessibility may not necessarily account for Zn bioaccessibility.

Although phytate reduction in pea proteins was found to increase the intestinal solubility of fortified Fe in Chapter 4, the final experiment in Chapter 7 demonstrated some trade-offs between improved Fe bioaccessibility and effects on a human gut microbiota *in vitro*. The findings reaffirmed the existing literature reporting that iron fortification or supplementation leads to increased delivery into the colon, which led to altered patterns of SCFA production and compositional shifts in the microbiota favouring the iron scavenging *Enterobacteriaceae* family (Yilmaz & Li, 2018b). This effect from Fe was partially mediated in the presence of the pea protein isolate (PPI) but not pea protein hydrolysate (PPH), where only fortified PPI increased total SCFAs production, corresponding with greater abundance of SCFA producers (e.g. *Propionibacteriaceae*) relative to the Fe control. The protective effect was observed despite PPI presenting additional soluble colonic Fe relative to PPH, signifying the presence of protective factors or structures in PPI that was lost during enzymatic hydrolysis. Additionally, the study identified that Fe fortification led to increased colonic sulfur delivery from endogenous proteins, which may have been one factor potentiating the negative effects of Fe on the colonic microbiota. The mechanism may be similar to that from dietary sulfur-containing amino acids, where iron-

rich sources such as meats have been shown to be a major substrate for sulfide generation in the lower gut (Magee, Richardson, Hughes, & Cummings, 2000).

Given the imperative role of legume seeds as a complementary source of protein in plant-based diets, the outcomes from these studies impel broader implications for the design of nutritional strategies targeting Fe and Zn deficiency. The effect of element fortification on enhancing protein-protein and protein-phytate interactions should be actively considered, particularly as phytate is distributed throughout the seed in legumes. Such associations can begin pre-harvest from seed maturation to post-harvest during food processing, and ultimately gastrointestinal digestion. This suggests that both pre- and post-harvest approaches to enhance element density can be affected by its complex interrelationship between phytate and protein, altering the efficacy of strategies such as biofortification. While modification of phytate content in seeds are often accompanied by trade-offs in field performance and seed quality (Raboy, 2020), there are ample opportunities to modulate these interactions towards increased protein solubility prior to dietary ingestion, as demonstrated through enzymatic hydrolysis in this study.

8.2. Directions and Outlook

This series of investigations spark a new paradigm for fortification strategies targeting Fe and Zn bioaccessibility through legume matrices. Provided the critical role of soluble peptides and amino acids as critical determinants of Fe and Zn solubility, attempts to enhance their bioaccessibility in legume matrices should be repositioned to consider protein quality. Processes and/or ingredients that improve aspects of protein digestibility, amino

acid composition, and ultimately bioavailability are all intertwined to interact with elements during extraction, processing, and digestion. In addition to a reduction of anti-nutritional factors such as phytate, changes to protein structure-digestibility relationships may be attained by additives such as NaCl. At low concentrations, both sodium and chloride ions can increase protein solubilisation by decreasing protein-protein interactions during extraction (Dumetz, Snellinger-O'brien, Kaler, & Lenhoff, 2007). The two ions can further compete with Fe and Zn to reduce the availability of residual phytate and endogenous phosphates during digestion (Zhang et al., 2020). However, some honing is required to avoid excessive ionic strength that destabilises proteins, which would hinder enzymatic hydrolysis within the lumen.

Towards the direction of exploring the Fe-binding pea peptides identified in this study as individual ingredients for supplementation or fortification, it would be important to optimise the conditions required for each peptide. Such optimisation is essential to achieve a moderate level of chelation that leads to eventual dissociation. Factors such as iron form, iron:peptide mass ratio, and other binding conditions (e.g. pH and holding time) should be examined in conjunction with its pharmacokinetic behaviour, using simulations of intestinal lumen conditions. It is acknowledged that the original source of the food peptides is also a determinant of its physicochemical properties during digestion affecting Fe bioaccessibility. Targeted peptide purification can enhance its Fe-binding capacity and eliminate undesirable compounds, such as negatively charged phosphate groups that make adjacent peptide bonds proteolysis-resistant (Hu, Lin, He, & Sun, 2022). However, there may also be potential loss of other constituents within the food matrix that may work in concert towards

bioavailability. For example, it has been found in barley glutelin hydrolysates that while lower MW peptides (< 1 kDa) are the most effective at Fe-chelation, the presence of large MW peptides (> 10 kDa) contributed to reducing power (Xia et al., 2012). In such context, co-administration of the peptide mixture may produce the optimal effects on enhancing Fe bioavailability.

Given the highly saturable nature of the carrier-mediated duodenal uptake, the majority of dietary Fe arrive at the lower gut, where there has been compelling evidence of microbial crosstalk regulating host Fe homeostasis (Botta et al., 2021). As such, the gut microbiota remains a relevant therapeutic target for mediating Fe absorption, both directly and at a systemic level. The use of prebiotics that stimulate the production of SCFAs and organic acids can lead to localised lowering of gut pH, which may increase Fe bioaccessibility (Ahmad et al., 2021). Whilst some species with high-affinity acquisition systems tend to make Fe unavailable to the host, some commensal bacteria can enhance Fe bioaccessibility in the form of diffusible siderophores that can be taken up by the host proteins such as lipocalin (Seyoum et al., 2021). Moreover, the immunomodulatory and anti-inflammatory properties of probiotic species can mediate Fe absorption *via* hepcidin regulation (Zakrzewska et al., 2022). These mechanisms bring to light new possibilities for innovative approaches to enhancing Fe bioavailability, whilst potentially attenuating some of its negative ramifications on the gut microbiota. Such an innovative technique was investigated by Garcés et al. (2018), who were able to deliver iron oxide nanoparticles into gut enterocytes through *Lactobacillus fermentum* as a carrier.

Several limitations of the studies undertaken in this thesis should be addressed in future investigations, such as the use of solubility as a proxy for bioaccessibility through static *in vitro* gastrointestinal models. This approach benefited the study by eliminating confounding effects from inter-individual variation in the digestion stages prior to colonic fermentation, and replicating the inhibitory effects of legume proteins on Fe bioavailability as previously observed *in vivo*. However, it is recognised that whilst solubility is a prerequisite for nutrient absorption, not all soluble complexes tend to be bioavailable for mucosal transfer into the circulation and utilisation by the body (Fairweather-Tait, 2002). Additionally, true bioaccessibility is also inclusive of the fraction present on the surface of the particulates (Apostoli, 2006), which was likely not accounted for as centrifugal separation was used to obtain the soluble fraction. The discrepant results in iron solubility observed between different *in vitro* gastrointestinal models in Chapter 7 compared with Chapter 5 revealed that varying model parameters (e.g. ionic strength from digestion fluids) can be an additional determinant of solubility as a bioaccessibility indicator. Given the biological relevance of food peptides in modulating Fe and Zn intestinal absorption regulators (e.g. DMT-1, hepcidin) *in vivo (Thomas et al., 2013)*, follow-up studies through animal models or human trials should be conducted to create a broader picture of the ADME properties from these treatments.

The current research would also benefit from replications *in vitro*, where speciation studies on the insoluble particles formed during each phase of digestion would uncover the nature of interactions causing aggregation in the lumen. Instead of an end-point approach to sampling, the kinetics of Fe and Zn aggregation could be monitored as a function of time

points. Such understanding would assist in the design of food structures and molecules that keep these elements in solution through controlled release during small intestinal digestion, such as protein hydrolysates. In the case of Fe, it would be insightful to examine changes in oxidation state throughout the digestive tract, as this would provide a snapshot of its redox activity in the presence of antioxidant ligands such as proteins. These findings would be complemented by concurrent analyses of some siderophores released during colonic fermentation (e.g. Enterobactin), which are also indicators of protection from oxidative stress (Peralta et al., 2016).

Micronutrient deficiency remains a pressing systemic issue underpinned by global socioeconomic inequality, of which poor nutritional access is merely one constituent. Fe and Zn deficiencies have been specifically proposed as a common repercussion of the global triple burden of undernourishment, hidden hunger, and obesity from increased consumption of ultra-processed foods (Freeland-Graves, Sachdev, Binderberger, & Sosanya, 2020). Populations most vulnerable to these conditions include children and women in low- and middle-income communities, whom are the most profoundly impacted by the functional consequences of low intakes in bioavailable Fe and Zn, often since birth. Here, the problem transcends the lack of diversified diets promoting absorption, but systemic limitations in homeostasis (e.g. from inflammation or infection), and poor health literacy. This perpetuates vicious cycles where impaired bodily growth and function continues to fundamentally limit productivity beyond individual scales, broadening the existing disparity in social and income equality. Element supplementation or food fortification practices have a crucial role in empowering disadvantaged populations at the

physical health level, given that strategies are designed in consideration of extant food practices within target communities that can affect bioavailability. To genuinely alleviate the pedigree of micronutrient deficiency as a public health dilemma, the literary character Dirk Gently epitomised it best when he said, "...what we are concerned with here is the fundamental interconnectedness of all things" (Adams, 1988).

9. References

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10. Appendices

Supplementary Data 1

Table 20. Some chelation properties of polyphenols or carotenoids in relation to binding and/or bioavailability of divalent cations

Chelation properties		Additional notes	Source
Chelating sites	Deprotonated phenolic -OH	Proton displacement under physiological pH (pH 5-8). Phenoxide group favours interaction with $Fe^{3+/2+}$ and Zn^{2+} but not alkaline earth metals (i.e. Ca^{2+})	(Andjelković et al., 2006; Hider et al., 2001; Kasprzak, Erxleben, & Ochocki, 2015)
	Pyrone Oxygen	Prominent in non-fused vs. fused rings	(Hider et al., 2001)
	-COOH and -$COOCH_3$	-$COOCH_3$ is a stronger electron donor than –COOH	(Radalla, 2015; Yang et al., 2014)
	Glycosylation	↑ total number of –OH groups, but ↓ dissociable proton from phenolic backbone ↑ aqueous cellular solubility	(Cherrak et al., 2016; Kumar & Pandey, 2013)
Chelating positions	Consecutive –OH groups	↑ coordination from ortho position on same ring (e.g. in catechol and galloyl moieties) or peri position on two fused rings ↑ Number of –OH groups also ↑ denticity	(Andjelković et al., 2006; Zhang & Allen, 1995)
Properties of aromatic ring substituents	Long alkyl chains or –CH_3	↑ Lipophilicity ↑ coordination sites	(Kontoghiorghes, 1982)
	All electron donor groups	↑ basicity of coordinating group > ↑ complex stability	(Kontoghiorghes, 1982; Lundgren & Stradiotto, 2016)

Table 21. Composition of the simulated salivary medium used in Chapter 4

Element (MW g/mol)	Salt compound (MW g/mol)	Amount in d.water (g/L)	Equiv. (mmol/l, mg/l or g/l)	Equiv. Element conc.	Reference value range (mmol/l)
Na (22.99)	NaCl (58.44)	1.5	25.4667 (mM NaCl/l)	10.1 (mM Na/l)	2-22[1] 11.5-217.3[3] 5.2-11.5[4] 11.5–18.6[5]
Ca (40.078)	$CaCl_2 \cdot 2H_2O$ (147.0146)	0.2	1.3604 (mM CaCl2·2H2O/l)	0.371 (mM Ca/l)	0.47-0.52[2] 0.20-0.28[4] 0.23–0.37[5]
K (39.098)	KH_2PO_4 (136.086)	3	22.045 (mM KH2PO4/l)	6.334 (mM K/l)	6.4-37[1] 2.6-18.3[3] 2-26[4] 25.1–34.7[5]
P (30.974)	KH_2PO_4 (136.086)	3	5.423 (mM KH2PO4/l)	5.018 (mM P/l)	0.9-7.75[4] 5.10–8.78[5] 8.55-11.16[8]
Cl (35.453)	$CaCl_2 \cdot 2H_2O$ (147.0146)	0.2	1.3604 (mM CaCl2·2H2O/l)	0.656 (mM Cl/l)	5-40[1] 10-13.5[9] 2.34-7.55[10]
	NaCl (58.44)	1.5	25.6674 (mM NaCl/l)	15.57 (mM Na/l)	
Proteins	Total (g/l)				1.1-1.8[1] 1.9-2.1[2] 2.73-3.22[6] 0.5-1.5[12]
	Mucin (g/l)	1.5			1.02-1.36[6] 1.78-2.02[7]

Element (MW g/mol)	Salt compound (MW g/mol)	Amount in d.water (g/L)	Equiv. (mmol/l, mg/l or g/l)	Equiv. Element conc.	Reference value range (mmol/l)
	Amylase (U/l)	20000			11900-304700[1] 71460-82960[6] 78630-100630[7]
	Albumin (mg/l)	200			246-344[1] 580-740[11] 0-200[12] 26.1-102.9[13]
	Urea (mg/l)	150			170-410[1] 50-480[4] 125-285[14]

[1]Biochemistry standard reference range (Carda, Mosquera-Lloreda, Salom, Gomez de Ferraris, & Peydró, 2006), [2,3,4,5,6,8,9,10,11,12,13,14]Unstimulated saliva *in vivo* (Ambikathanaya, Hegde, & Ayas, 2018; Gonçalves et al., 2019; Henskens, van der Velden, Veerman, & Amerongen, 1993; Kallapur et al., 2013; Kejriwal, Bhandary, Thomas, & Kumari, 2014; Labat et al., 2018; Lasisi, Raji, & Salako, 2016; Lima-Aragão et al., 2016; Metgud & Patel, 2014; Sánchez, Miozza, Delgado, & Busch, 2011; Savica et al., 2008; Shirzaiy, Heidari, Dalirsani, & Dehghan, 2015; Vaziri et al., 2009), [7]Stimulated saliva *in vivo* (Sánchez et al., 2011)

Supplementary Data 2

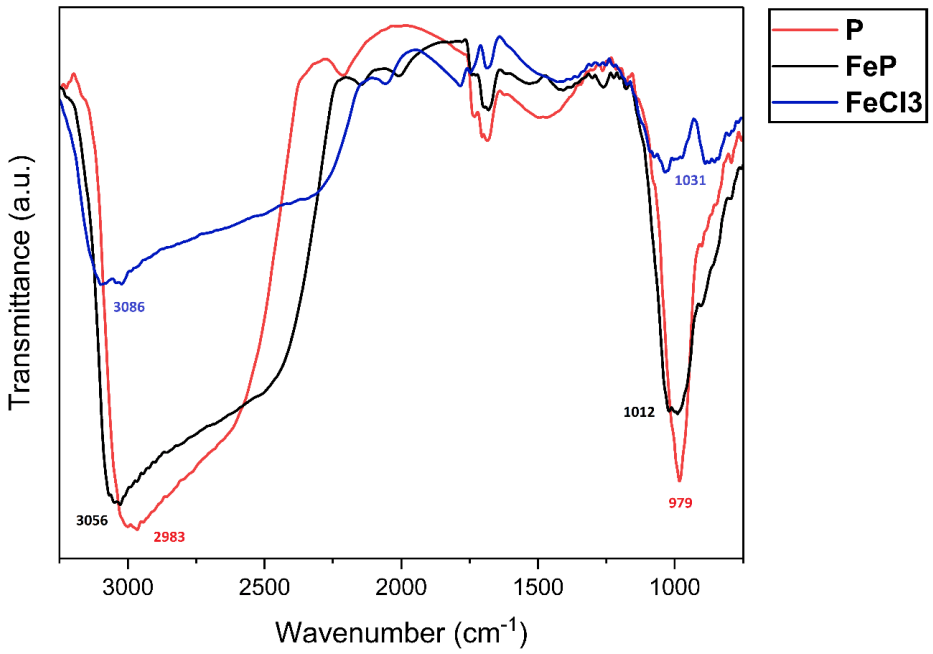
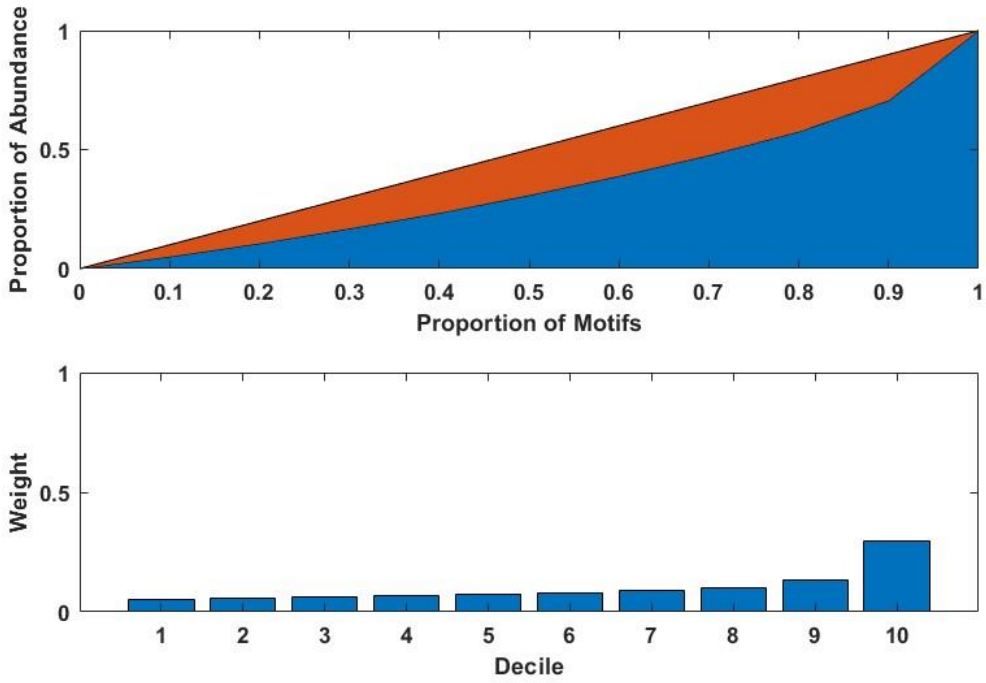
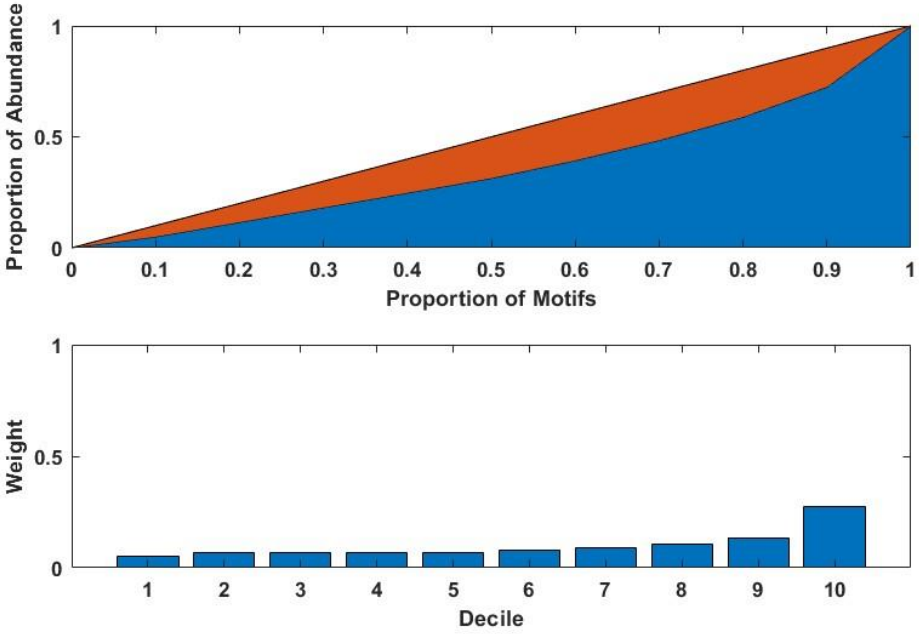


Figure 24. Fourier transform infrared (FTIR) spectra of phosphate elution buffer (P), iron solution ($FeCl_3$) and buffer + iron solution (FeP).

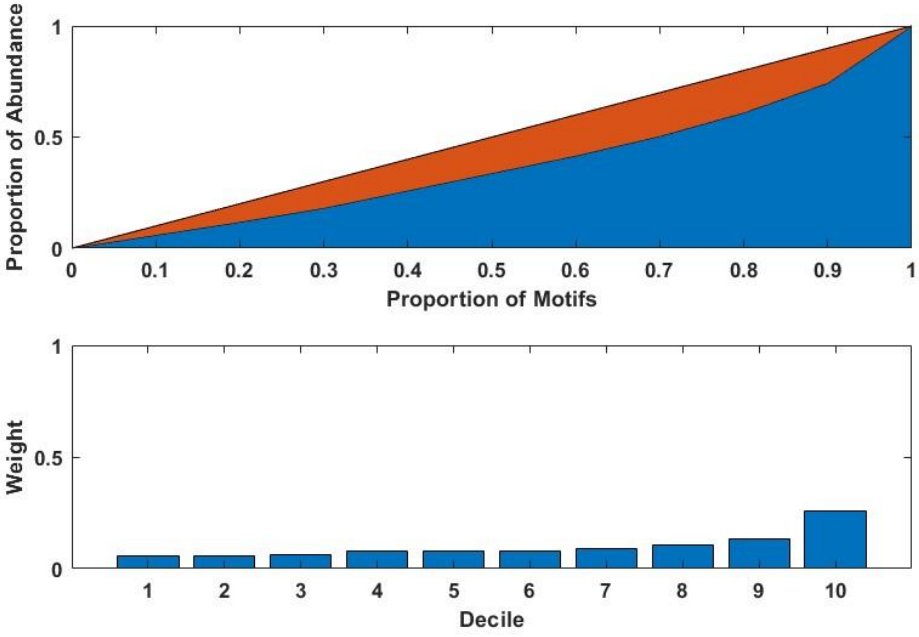
a)



b)


Proportion of Abundance
1
0.5
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Proportion of Motifs
Weight
1
0.5
0
1
2
3
4
5
6
7
8
9
10
Decile

c)


Proportion of Abundance
1
0.5
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Proportion of Motifs
Weight
1
0.5
0
1
2
3
4
5
6
7
8
9
10
Decile

d)

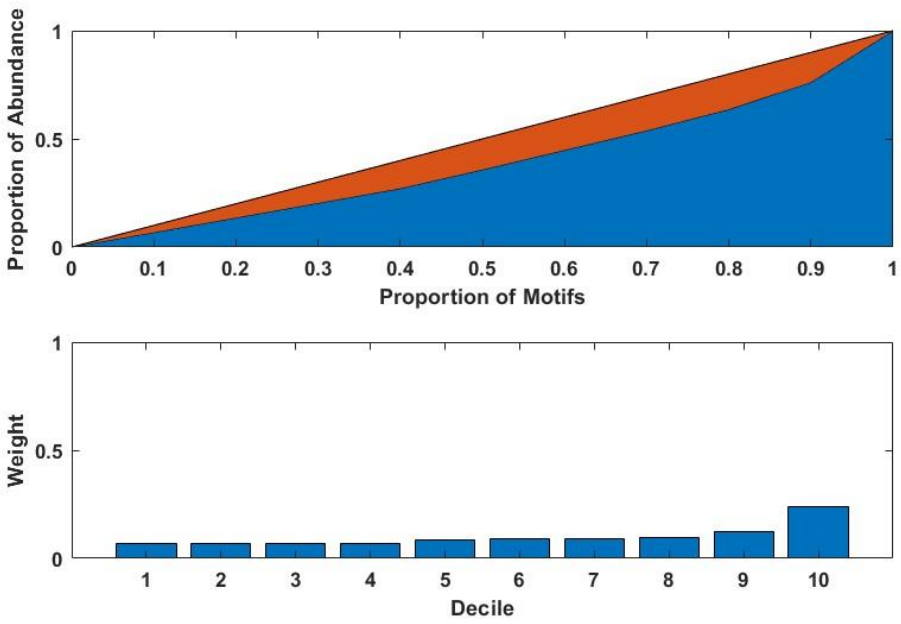


Figure 25. The approximate Lorenz Curve (top) and concentration distribution by deciles (bottom) generated for each sequence length of the potential iron-binding motifs: a) 3-mer, b) 4-mer, c) 5-mer and d) 6-mer.

Supplementary Data 3

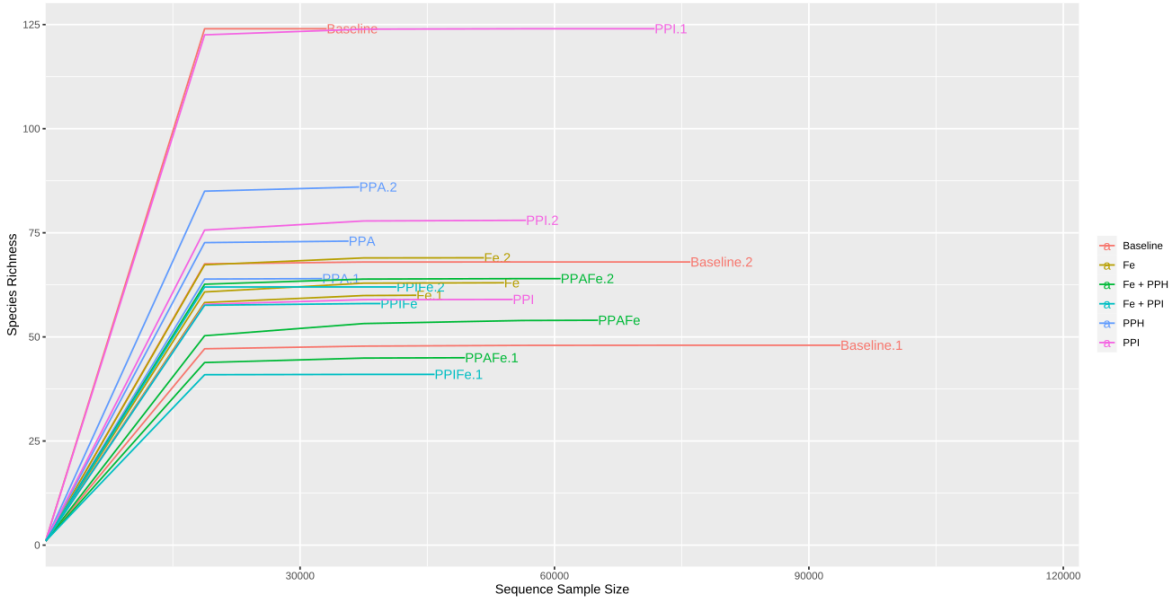


Figure 26. The rarefaction curve generated from 16SrRNA analyses in Chapter 7.