

THESIS FOR THE DEGREE OF DOCTOR IN TECHNOLOGY

# Nutritional Limitations of a Green Protein Shift with Focus on Iron

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## ABSTRACT

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A dietary shift into plant-based diets (PBD) to reduce the climate footprint is advocated. Effects on nutrition and health from a modern PBD, composed of replacement products based on protein extracts are however currently unknown. In Scandinavian countries, where cultivation of soy is not suited to the climate, domestic crops such as fava bean have been suggested as having large potential in replacing animal products. In this thesis, fava bean has therefore been selected as a model crop for investigating the replacement of meat in the context of a green protein shift.

Analyzing 15 cultivars of fava bean, only one had an adequate estimated bioavailability of iron, and variation in antinutritional contents (phytate, lectins, trypsin inhibitors, oligosaccharides, saponins, total phenolics) was high. Even greater variations were seen in antinutritional and nutritional contents of 42 meat substitutes on the Swedish market, probably explained by differences in processing methods and the accumulation of phytate by protein extraction. Meat substitutes had a high content of phytate and a very low estimated bioavailability of iron and zinc, except for mycoprotein products and tempeh. Also, a high content of salt, saturated fat and dietary fiber was found in meat substitutes.

Low theoretical iron bioavailability of extracted and texturized fava bean protein was confirmed in a clinical study. Non-heme iron absorption from single meals in 27 women of fertile ages measured with radioisotope technique was 4.2% from texturized fava bean meal, 21.7% and 9.2% from beef and cod protein meals respectively.

Furthermore, fermentation of a fava bean drink for decreasing phytate content, and thereby improve iron and zinc bioavailability, was investigated. A *Pediococcus pentosaceus* strain was able to substantially degrade phytate during co-fermentation with a phytase-producing yeast strain. By itself, *P. pentosaceus* did not have the ability to degrade phytate, which illustrates the importance of optimal conditions as well as selection of suitable microorganisms during fermentation for improved bioavailability.

Overall, results show that nutritional consequences of consuming meat substitutes and fava bean is significantly affected by the product, cultivar and processing method used. Substituting meat with products analyzed in this thesis is estimated to markedly reduce the absorbable iron from the diet.

**Keywords:** fava bean, protein shift, plant-based, iron bioavailability, non-heme iron absorption, zinc bioavailability, protein extract, meat substitutes, sustainable nutrition, antinutrients, phytate



## LIST OF PUBLICATIONS

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This doctoral thesis is based on the work contained in four papers:

- I. **Inger-Cecilia Mayer Labba**, Hanne Frøkiær, Ann-Sofie Sandberg. Nutritional and antinutritional composition of fava bean (*Vicia faba* L. var. minor) cultivars). *Food Research International*, 2020. 140: 110038.  
doi: <http://doi.org/10.1016/j.foodres.2020.110038>
- II. **Inger-Cecilia Mayer Labba**, Thomas Andlid, Åsa Lindgren, Ann-Sofie Sandberg, Fei Sjöberg. Isolation, identification, and selection of strains as candidate probiotics and starters for fermentation of Swedish legumes. *Food & nutrition*, 2020. **64**: 4410.  
doi: <http://doi.org/10.29219/fnr.v64.4410>
- III. **Inger-Cecilia Mayer Labba**, Hannah Steinhausen, Linnéa Almius, Ann-Sofie Sandberg. Nutritional composition and estimated iron and zinc bioavailability of meat analogues available on the Swedish market. (*Submitted*)
- IV. **Inger-Cecilia Mayer Labba**, Michael Hoppe, Elisabeth Gramatkovski, Martin Hjellström, Mehdi Abdollahi, Ingrid Undeland, Lena Hulthén, Ann-Sofie Sandberg. Lower non-heme iron absorption in healthy females from single meals with texturized fava bean protein compared to beef and cod protein meals: two single-blinded randomized trials. *Nutrients*, 2022. 14(15), 3162.  
doi: <https://doi.org/10.3390/nu14153162>

Other papers, not included in this thesis:

- Amanda Helstad, Erica Forsén, Cecilia Ahlström, **Inger-Cecilia Mayer Labba**, Ann-Sofie Sandberg, Marilyn Rayner, Jeanette K. Purhagen. Protein extraction from cold-pressed hempseed press cake: From laboratory to pilot scale. *Journal of Food Science*,

2021. **87**: 1.

doi: <https://doi.org/10.1111/1750-3841.16005>

- Cecilia Ahlström, Johan Thuvander, Marilyn Rayner, Inger-Cecilia Mayer Labba, Ann-Sofie Sandberg, Karolina Östbring. Pilot-Scale Protein Recovery from Cold-Pressed Rapeseed Press Cake: Influence of Solids Recirculation. *Processes*. 2022  
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## CONTRIBUTION REPORT

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**Paper I:** Inger-Cecilia Mayer Labba (ICML) participated in planning and conceiving the study, performing chemical analyses, interpreted the data and wrote the first draft of the manuscript.

**Paper II:** ICML conceptualized the study, performed the laboratory work, data analysis and wrote the first draft of the manuscript.

**Paper III:** ICML conceptualized and planned the study, supervised Linnéa Almius and Hannah Steinhausen who performed major parts of the laboratory work. ICML interpreted data and wrote the first draft of the manuscript.

**Paper IV:** ICML participated in planning and conceiving the study, produced test meals, recruited participants for the clinical trial, performed chemical analysis of meals, interpreted the data and wrote the first draft of the manuscript.

## ABBREVIATIONS

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BSH	Bile Salt Hydrolase
CFU	Colony Forming Unit
DHA	Docosahexaenoic Acid
DW	Dry Weight
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Eicosapentaenoic Acid
FAO	Food and Agriculture Organization
FAME	Fatty Acid Methyl Esters
GC-MS	Gas Chromatography – Mass Spectrometry
GHG	Greenhouse Gas
HPIC	High Pressure Ion Chromatography
InsP <sub>n</sub>	Inositol Phosphates, with <i>n</i> phosphate groups
LAB	Lactic Acid Bacteria
LC-MS	Liquid Chromatography – Mass Spectrometry
LDL	Low Density Lipoprotein
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass-Spectrometry
MRS	Man, Rogosa and Sharpe
MUFA	Monounsaturated Fatty Acids
NNR	Nordic Nutrition Recommendations
PBD	Plant-Based Diets
PUFA	Polyunsaturated Fatty Acids
rep-PCR	Repetitive – Polymerase Chain Reaction
RFOs	Raffinose Family Oligosaccharides
ROS	Reactive Oxygen Species
SDGs	Global Sustainability Goals
UN	The United Nations
WBC	Whole Body Counting
WHO	World Health Organization



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## 1. INTRODUCTION

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It is indisputable that global action is urgently needed to limit global warming, and during recent decades climate change has been given an immensely increased focus. In 2015, 196 countries agreed on the targets set in The United Nations (UN) Paris Agreement to limit global temperature rise to a maximum of 2°C above pre-industrial levels [1]. Since the 2006 report by the Food and Agriculture Organization (FAO) of the UN “Livestock’s long shadow” [2], stating that livestock accounts for 18% of the global anthropogenic greenhouse gas (GHG)-emissions, a reduction of industrially produced meat from ruminant animals has been seen as a major measure for the restriction of climate change [3-7]. The magnitude of the contribution of GHG-emissions from livestock has since been revised by the FAO [8], and been heavily debated since agricultural systems are complex and have the ability to both sequester and emit CO<sub>2</sub> [9-11]. The shift from carbon-equivalent heavy protein sources, mainly from ruminant livestock, into protein sources considered less climate burdensome has come to be known as the protein shift [12].

While I acknowledge that the protein shift includes changes and effectivization of production systems, side stream utilization, increased circularity and several more aspects, I use the definition in this thesis to refer to an increase of plant foods such as pulses, grains and vegetables, which are being highlighted as low contributors to GHG-emission, and a decrease of foremost products from ruminant livestock. The food sector has been widely discussed as a major target for reducing GHG-emissions and reaching the Paris Agreement. Investigation of the effects by the protein shift on human health has mainly revolved around a high-quality plant-based diet, rich in whole grain, vegetables, pulses, nuts and seeds. While such a diet has long been associated with lower incidence of a number of metabolic diseases, it has also been questioned as not being a realistic food pattern across Western populations [13, 14], and been connected to certain nutritional shortcomings [15]. Modern plant-based food patterns, comprising meat substitutes based on protein extracts, as well as refined food products, are increasing in popularity [16, 17]. Nutritional and antinutritional composition and quality of such diets, bioavailability of nutrients, or their potential health implications have not been as thoroughly discussed in the context of the protein shift even though the nutritional limitations and potential risks associated with a plant-based diet has been known for decades.

## 2. OBJECTIVES

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The overall goal of this thesis was to investigate the nutritional and antinutritional composition of plant-based food products aimed at replacing meat, study strategies for improved nutritional quality and compare a plant-based model meal with meat and fish with respect to iron absorption. In this thesis, locally produced fava bean was used as a model crop for the replacement of meat in the context of a green protein shift.

To achieve this goal, the specific aims were to:

- Analyze the content of nutrients, antinutrients and estimate iron and zinc bioavailability in different varieties of fava bean
- Examine/investigate the potential use of fermentation as a method to substantially diminish the content of phytate to increase the bioavailability of iron and zinc in fava bean
- Assess the nutritional composition, content of relevant antinutrients, and estimate the bioavailability of iron and zinc in common meat analogues found on the Swedish market
- Evaluate the absorption of non-heme iron in women from a meal of texturized fava bean protein compared to a meal of beef protein and a meal of cod protein.
- Estimate the implications for women of fertile ages when substituting meat with texturized fava bean protein and other meat analogues. Calculate how it affects to meet the iron needs.

### 3. BACKGROUND

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#### *3.1 Nutritional aspects of a green protein shift*

While the magnitude, and strength of evidence, between red meat and metabolic diseases are under current debate [18-21], a diet rich in whole grain, dietary fiber, pulses, vegetables, nuts and seeds has long been associated with positive health effects [22-24]. Mainly plant-based diets have also been highlighted as having low climate footprints and has been suggested to be healthy and economically favorable as the prevalence of non-communicable diseases has been estimated to decrease with such a dietary shift [25, 26]. For example, in the 2019 FAO and World Health Organization (WHO) joint report on guiding principles for sustainable healthy diets, emphasis was put on a low-meat diet [27]. While many individual plant-based foods have been related to negative health effects, such as added sugars, starchy foods, refined carbohydrates, crisps and French fries [28-30], the association between the quality of plant-based diets and disease has until recently been very limited. Differences between a mainly whole-foods, plant-based diet compared to a lower quality plant-based diet containing refined food products have started to receive increasing interest. A lower quality plant-based diet has been associated with an increase of hypertension [31], lipid disorders [32], type 2 diabetes [33], abdominal obesity and high fasting glucose in women [34], coronary heart disease [35], the metabolic syndrome, hypertriglyceridaemia and mortality [36-38].

The use of refined plant-based products aiming at replacing meat and other animal products is becoming increasingly common [39]. Emerging studies have shown that meat substitutes, frequently based on legume and cereal protein extracts, often is produced with a high content of energy, salt and saturated fat, lacking the same amount of fiber as the health-promoting whole crop [40-43]. Plant-based alternatives to ground beef in the US has been shown to contain a level of iron and zinc that can be compared to ground beef, but they also contain high amounts of phytate that inhibit mineral absorption [40].

Since mineral content in itself does not reflect minerals absorbed from plant foods, and data on bioavailability from meat substitutes are lacking, it is difficult to compare the nutritional quality and value of these substitutes compared with meat [40].

Despite new findings, there is a strong perception among the general population that plant-based foods *per se* are healthy and sustainable [44]. There is a wide variety of dietary patterns comprising a large, or complete, portion of plant foods. Plant based food patterns can be divided

into subgroups, depending on the level of exclusion: Semi-vegetarians, or flexitarians, who consume a limited amount of meat and fish; pescatarians, who exclude all meats except for fish; lacto-ovo vegetarians who exclude all meats and fish and lastly strict vegetarians or vegans who exclude all meats, fish, dairy, eggs and other animal products such as honey [45]. A flexitarian diet has no formal definition, and its dietary framework remains open for interpretation with regards to meat reduction. A suggested limit for the definition of flexitarian diet is consumption of maximum one serving of meat and fish once per week [46, 47]. In this thesis, plant-based diets are referred to as dietary pattern that have totally, or to a large extent, excluded animal products from the diet.

A plant-based diet, regardless of quality, has been associated to an increase of certain nutritional deficiency risks. These risks are connected to a low intake, and/or uptake, of calcium, iodine, iron, zinc, selenium, vitamin B12 and vitamin D [15]. Additionally, intake of docosahexaenoic acid (DHA) and eicosatetraenoic acid (EPA), which are mainly found in fish and seafood, have been shown to be inadequate in pure vegetarian and vegan diets [48, 49]. Observational studies have found an increased prevalence of low iron stores, iron deficiency [50-52], low intake and status of vitamin D, vitamin B12, zinc, iodide and calcium [53], lower bone mass density [54-56] and bone fractures [57] in vegetarians and vegans compared to omnivores. Lower iron status among vegetarians has mainly been explained by the lower bioavailability of iron in plant foods. Meanwhile, total iron intake has been reported to be higher among plant-based food patterns [58-61].

This illustrates the complexity of sustainability, which consist of many different aspects that sometimes stand in conflict with each other. The risk of focusing on a single aspect of sustainability is that other aspects, such as health and nutrition, will be sidelined and deteriorate.

### *3.2 Iron deficiency*

Iron deficiency is the most common and widespread nutritional disorder in the world, affecting approximately 30% of the global population [62]. Among women of fertile ages living in Western countries, the prevalence of iron deficiency has been estimated to 10-30% [63-66]. Among Swedish teenage girls in the 8<sup>th</sup> grade of elementary school, 30% are estimated to suffer from iron deficiency [67]. Iron deficiency, and iron deficiency anemia, can be caused by insufficient iron intake from the diet or by poor absorption [68]. Since women across Western

societies are twice as likely as men to follow plant-based diets [69], this is a population group particularly vulnerable of developing low iron status and iron deficiency since plant-based diets have low bioavailability of iron. Deficiency of iron and iron deficiency anemia can cause poor physical and mental performance, lower productivity, cognitive developmental defects and delay motor development in children. The negative impact on cognitive development in children might also have lasting effects later in life, even though the iron deficiency anemia is treated [70-73]. Severe iron deficiency during pregnancy has been connected to an increased risk of preterm-labor, low neonatal weight and newborn mortality [74]. Preventing iron deficiency is therefore important for public health.

### *3.3 Iron absorption and metabolism*

Iron is an essential trace metal to humans, responsible for mitochondrial function, DNA synthesis and repair, production of new red blood cells as well as many enzymatic reactions, and together with hemoglobin responsible for transportation of oxygen in the blood [75]. The human body store iron as ferritin and hemosiderin mainly in the liver, spleen and bone marrow [76]. Homeostasis of iron in humans is maintained through regulated absorption as well as a highly effective recirculation of iron from physiologically aged, senescent, red blood cells. Approximately 90% of the daily iron originates from senescent red blood cells recirculated by macrophages and made available by transportation back to the blood circulation [77]. The plasma entry is controlled by the regulatory hormone hepcidin that inhibits iron transportation into the blood stream when systemic iron levels are sufficient. When iron status is low, hepcidin levels decrease which allows iron to enter the systemic circulation [78]. Apart from high levels of circulating and tissue levels of iron, inflammation and infection can cause an elevated expression of hepcidin which limits the iron supply for erythropoiesis, causing an interruption of the iron homeostasis [77]. Overweight and obesity have been associated with an increase of mild iron deficiency, caused by chronic low-grade inflammation [79, 80].

Apart from individual physiological conditions such as iron status, dietary iron absorption can be affected by the food matrix as well as the type of iron present. In general, dietary iron is classified as either heme or non-heme [81]. Dietary heme iron is found in animal products mainly as part of hemoglobin and myoglobin, while non-heme iron is found in a variety of forms in both animal products and plant foods. Absorption of heme iron is little affected by dietary inhibitors and enhancers, apart from calcium in single meal studies, while the



absorption of non-heme iron can be greatly impacted by food components. Stimulating factors include ascorbic acid and unspecified factors from fermented vegetables as well as the meat factor, which is thought to be related to the protein, or peptide digestion products, of muscle tissue [82-85]. Phytate, calcium and certain polyphenols such as tannins are known to impair the non-heme iron absorption. The clinical relevance of inhibition of calcium has however been debated since long term intervention studies has not been able to prove any effects by calcium on iron status [86, 87].

### *3.4 Fava bean – a source of green protein*

The fava bean is a legume that, in contrast to the soy bean, can be cultivated in a wide variety of geographic locations, even in regions with a short growing season, such as the Boreal zone [88]. The fava bean has a long history of being used as food, with the oldest seeds of fava bean being traced to the late 10th millennium B.P. in north-west Syria [89]. In countries with more advanced economies, legumes are generally underutilized as food [90]. In Sweden, only 2.0% of the total cropland area was used for cultivation of legumes during the year of 2021 [91], with peas (55 900 tons from 22 040 ha) and fava bean (48 800 tons from 20 240 ha) as the two main legume crops cultivated [92]. The majority of fava bean and peas produced in Sweden are currently used as animal feed. In a scenario analysis by Rööf et al, it was estimated plausible to use 73 000 ha of the Swedish cropland for fava bean cultivation, utilized for food [93].

### *3.5 Nutrients and antinutrients in fava bean*

Legumes have favorable nutritional compositions for human consumption, being low in fat and high in protein, dietary fiber, iron, zinc and vitamins such as folate, riboflavin and thiamine [94]. Several health benefits have been observed in relation to the consumption of legumes, including reduced risk of colorectal cancer [95], improvement of gut health, reduced blood cholesterol levels [96], and reduced risk of cardiovascular disease [97, 98].

However, legumes also contain a number of bioactive compounds that are traditionally classified as antinutrients: phytate, saponins, lectins and protease inhibitors. These compounds may exert negative effects on the human body and can reduce the digestibility of nutrients [99, 100]. A plant-based diet that contain legumes often have a low bioavailability of minerals, owing to the presence of absorption inhibitors, iron-binding polyphenols and phytate [101, 102]. Thus, nutritional relevance of the high mineral content may be questioned. While lectins

and trypsin inhibitors from food matrixes can exert severe negative effects following intake, these compounds are easily inactivated by heat treatment [103, 104]. On the contrary, phytate is a heat stable molecule that has proven challenging to reduce to a level where it no longer interferes with mineral absorption [104].

Apart from the common antinutrients mentioned above, vicine and convicine are antinutritional compounds specific to fava bean. Upon ingestion of fava bean, these two compounds are hydrolyzed to divicine and isouramil which are two highly reactive redox compounds [105]. Divicine and isouramil are transferred into the blood stream where they produce reactive oxygen species (ROS) [106, 107]. In erythrocytes of healthy individuals, ROS are effectively detoxified by enzymatic reactions dependent on NADPH [108]. However, in individuals with the genetic disorder glucose-6-phosphate dehydrogenase deficiency, commonly named favism, NADPH is in short supply which leads to severe oxidative damage to erythrocytes. In the most severe cases, life-threatening acute hemolytic anemia can occur [109]. Apart from screening populations with a high prevalence of favism, which already is being done in Sardinia [110], plant breeding programs and developing processing methods for the decrease of vicine and convicine levels are important measures to reduce the negative impact of these compounds.

### *3.6 Protein extraction and processing*

The objective of extraction is to recover valuable components from a multitude of raw materials. Extraction of food fractions or nutrients are common procedures in the food industry [111]. Meat substitutes are commonly produced based on protein extracts [112]. Protein extraction, which is performed prior to extrusion, is known to affect the composition, quality and functionality of the raw material depending on the processing conditions applied. In general, there are two types of protein extraction methods, where wet processing techniques typically yield a more concentrated protein extract, meaning higher protein purity, compared with dry processing techniques. High purity protein isolates are not necessary or applicable for most food applications, as they in general have lower functionality compared to plant protein extracted by dry processing techniques [113]. Wet processing demands a higher energy use compared to dry processing, as well as a substantial amount of water, acid, base and other chemicals if used in combination with pretreatments [113]. Berghout et al estimated that production of lupine protein isolate using the wet processing technique pH shift, and a defatting

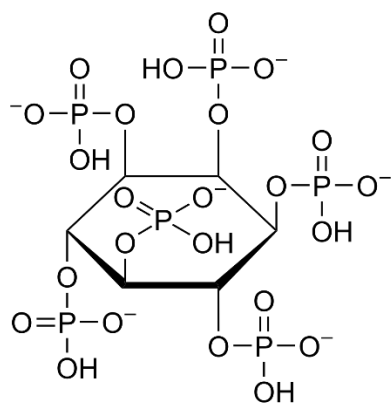
pretreatment, requires 80 L of water, 22.4 kg hexane, 40 g NaOH and 40 g HCl per kilo protein extracted [114]. Vogelsang-O'Dwyer et al conducted a life cycle analysis of fava bean protein extracted by air classification (64% protein, 1.07 kg carbon dioxide equivalents (CO<sub>2</sub>e)/kg protein) as well as for an extract using a patented wet extraction method (90% protein, 3.35 kg CO<sub>2</sub>e/kg protein) [115]. Heusala et al calculated the emitted CO<sub>2</sub>e from the production of dry fractionated fava bean concentrate (60% protein) in a scenario with high yield primary production, similar to yields reported in France and Germany. The emission was calculated to 1.9 CO<sub>2</sub>e per kilo protein [116]. In another scenario with low yield primary production, similar to reported yields from Spain and Italy, the emission was calculated to 3.4 CO<sub>2</sub>e per kilo protein which show differences between both environmental and climate impacts between processing methods. Another drawback of wet processing is the higher degree of protein denaturation and changes in protein functionality and digestibility, racemization of amino acids, loss of essential amino acids and protein hydrolysis [117-120].

Dry fractionated fava bean protein has been demonstrated to have higher solubility and greater gelling ability compared to wet extracted fava bean protein, as well as containing a higher level of sulfur containing amino acids [115]. On the other hand, dry processing is known to accumulate the antinutritional factors lectins, trypsin inhibitors, phytate and condensed tannins, while wet processed extracts with high purity have been shown to contain a lower content of antinutrients [121-125].

Extrusion cooking, which is a common method to create appealing texture of meat substitutes based on plant protein extracts, has been found to lower the content of phenolic compounds [126-128], affect amino acid composition [129], improve the digestibility of legume protein, reduce the content of trypsin inhibitors and hemagglutinin activity [130-132] and slightly reduce the phytic acid content [131] compared with the protein extract.

### *3.7 Phytate as an inhibitor of iron absorption*

Phytate, *myo*-inositol hexakisphosphate, Insp<sub>6</sub>, is abundant in plant foods such as legumes. The anion form is given in **Figure 1**. In legumes, phytate is found in seeds as storage molecules for phosphorus, energy, *myo*-inositol and cations [133]. Its chelating properties, enabling cation storage, is the reason for grouping phytate as an antinutrient since its negatively charged PO<sub>4</sub>-groups form insoluble compounds with dietary minerals (e.g. Ca<sup>2+</sup>, Mg<sup>2+</sup>, K<sup>+</sup>, Fe<sup>2+</sup>, Zn<sup>2+</sup>) at physiological conditions in the small intestine, inhibiting them from absorption by the human body.



**Figure 1. The hexavalent phytate anion**

The inhibitory effect of phytate on trace metal absorption is linked to the formation of insoluble and indigestible phytate-mineral complexes in the gut [134]. Phytates in the diet can lead to deficiencies of iron and zinc as the result of insufficient absorption [135]. When it comes to iron, absorption of heme iron is not vulnerable to inhibition by phytate, while the absorption of non-heme iron is impaired even at very low levels of phytate [136, 137]. The total content of minerals in a plant-based meal or product, where the content of inhibitory factors is high, is therefore not a good measurement for the contribution of dietary minerals without simultaneously evaluating the bioavailability.

### *3.8 Fermentation as a means to improve bioavailability of iron*

Fermentation can be performed by a large range of microorganisms, such as lactic acid bacteria (LAB) or yeasts. Lactic fermentation is one of the oldest methods for preserving foods. Fermentation has been shown to increase the nutritional value of foods, and intake of fermented foods are associated with a number of beneficial health effects, such as decreased postprandial blood glucose and insulin levels [138], reduction of cholesterol absorption [139], reduction of serum total cholesterol and low density lipoprotein (LDL) cholesterol [140]. Health benefits associated with fermented foods are often attributed to bioactive peptides that are synthesized in the microbial degradation of proteins by the microorganisms involved in the fermentation [141, 142]. Fermentation has also been shown to increase the bioavailability of nutrients by degradation of antinutrients and catabolism of macronutrients into more digestible compounds [143, 144].

Depending on the microorganisms and conditions, fermentation can reduce the content of phytate and tannins all of which are inhibitors of iron absorption. This reduction is a result of microbial enzymatic activity, as well as creation of conditions where plant endogenous enzymes are active [145] [146, 147]. For example, sourdough fermentation of bread and lactic fermentation of vegetables have been found to improve iron absorption in humans [148, 149]. Not all microorganisms express phytase, which is a phytate degrading enzyme, and the ability to reduce phytate during fermentation is varying even on strain level [150]. Therefore, fermentation as a method to reduce phytate require strain selection and optimal conditions, adapted to the present microorganisms as well as the raw material.

## 4. METHODS AND METHODOLOGICAL CONSIDERATIONS

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### *4.1 Selection of raw materials*

In study I, 15 cultivars of fava bean were used. Out of these, 14 originated from the same field, planned and harvested by Hushållningssällskapet in Grästorp, Sweden. These were the cultivars Banquise, Birgit, Boxer, Daisy, Fanfare, Fernando, Fuego, GL Emilia, Gloria, Lynx, Stella, Sunrise, Taifun and Tiffany. In addition, cultivar Alexia was harvested from a different field in the same region (Västra Götaland, Sweden).

The legume samples that were used in study II for isolation of LAB were cultivated on Öland, Sweden. A total of 23 samples were collected from which 12 were retrieved directly at the field and 11 from stored seeds.

Vegetarian meat analogues analyzed in study III were bought from two local food stores in Gothenburg city. A total of 44 samples were collected and included in the analyses.

In study IV, texturized fava bean protein was kindly provided by Vestkorn AS, Norway. The cod protein used in study III was extracted from filleting co-products that were provided by Fisk Idag AB and Sjöboden AB (Gothenburg, Sweden). The beef protein was extracted from boneless beef loins bought from a local retailer.

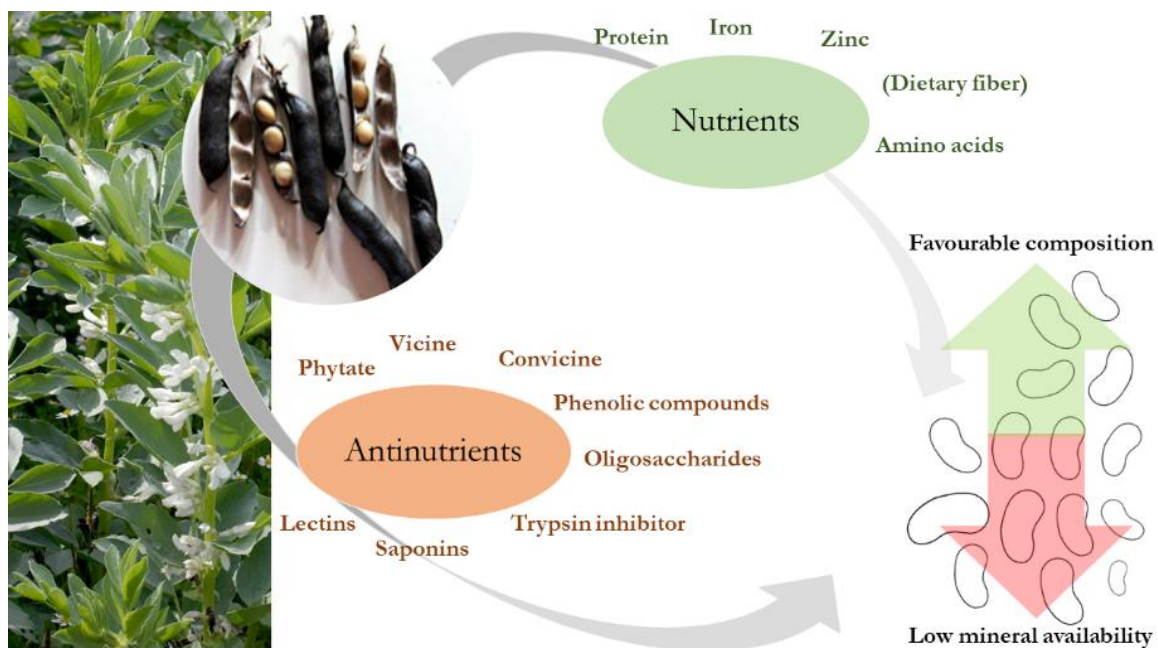
## *4.2 Study design*

This thesis is based on four studies. In study I, the nutrients and antinutrients of 15 different fava bean cultivars, harvested from the same region and growing season, were analyzed (**Figure 2**). Fe and Zn bioavailability were estimated by calculating the phytate:mineral molar ratio, i.e. the theoretical bioavailability according to [151].

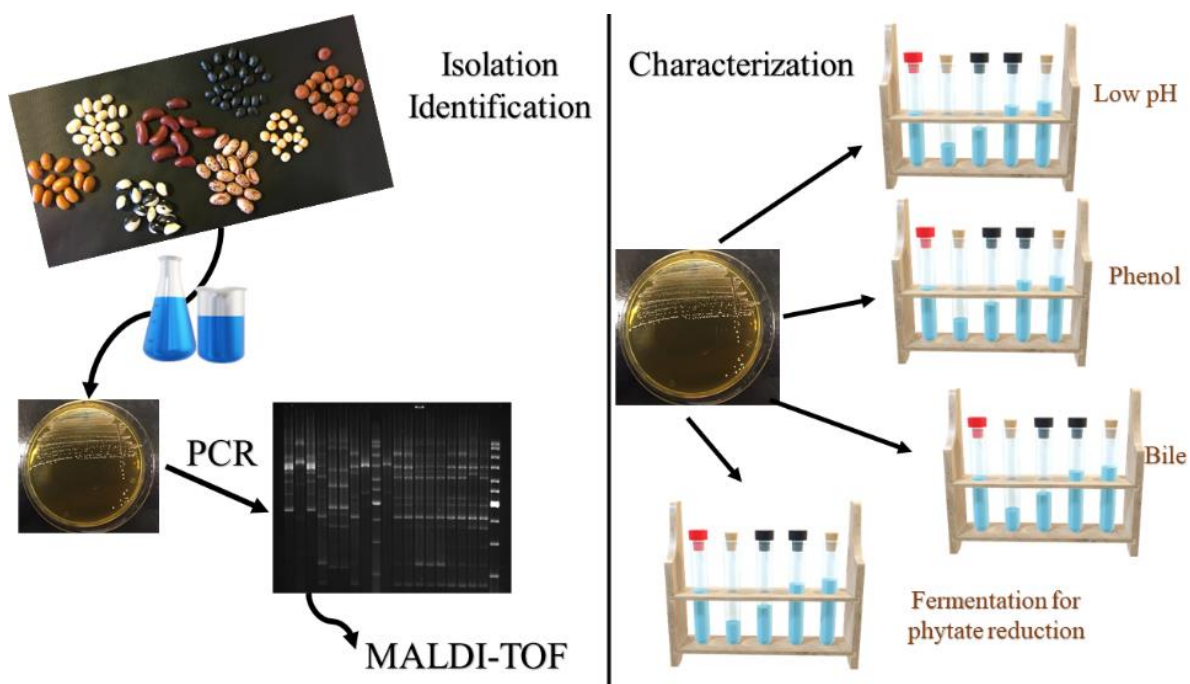
In Study II, we investigated the effect of fermentation by LAB and yeast on the level of phytate in a fava bean drink (**Figure 3**). The LAB were isolated from Öland-cultivated legumes. Isolates were also tested for their probiotic potential.

In Study III, we analyzed the nutritional content of 44 meat analogues commonly found in Swedish supermarkets (**Figure 5**). Antinutritional compounds phytate and total phenolic content was also measured. Bioavailability of Fe and Zn was estimated based on phytate:mineral molar ratio.

In Study IV, the non heme iron absorption from single meals based on texturized fava bean protein, cod protein and beef protein was investigated in two human clinical trials (**Figure 4**). The studies were performed in women of fertile ages and non heme iron absorption was measured by radioisotope technique. The trials were performed in collaboration with Sahlgrenska Academy, Gothenburg University, Sweden.

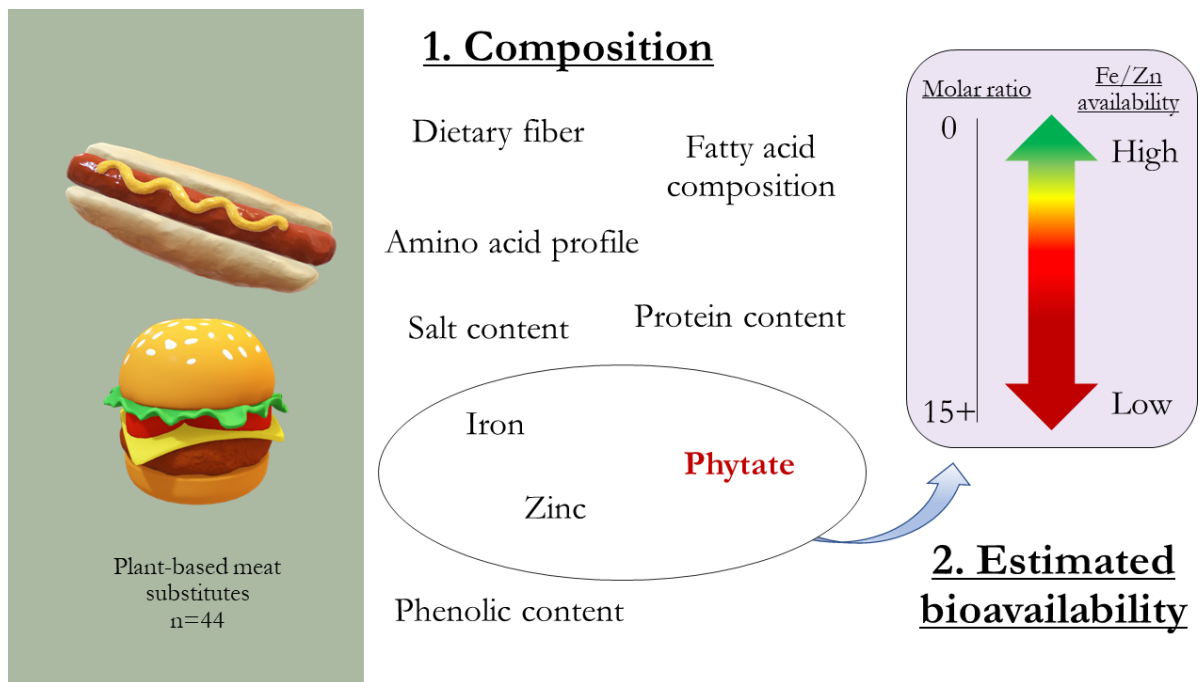


**Figure 2. Overview of paper I, Nutritional and antinutritional composition of fava bean (*Vicia faba* L. var. minor) cultivars)**

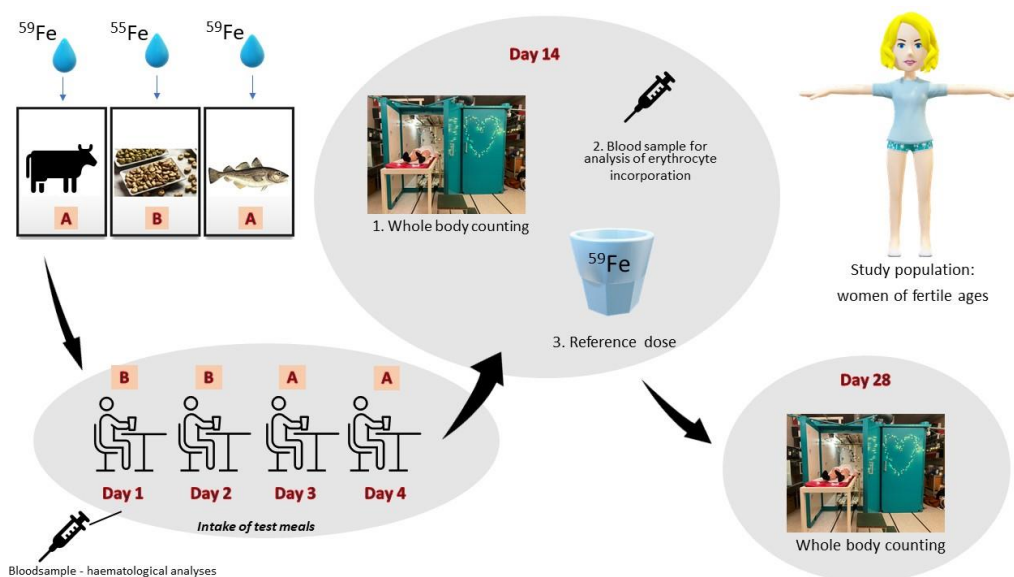


**Figure 3. Overview of paper II, Isolation, identification, and selection of strains as candidate probiotics and starters for fermentation of Swedish legumes**





**Figure 4. Overview of paper III, Nutritional composition and estimated bioavailability of Fe and Zn in meat substitutes on the Swedish market**



**Figure 5. Overview of paper IV, Lower non-heme iron absorption in healthy females from single meals with texturized fava bean protein, two single-blinded randomized trials: Trial 1: Fava bean protein vs beef protein; Trial 2: Fava bean protein vs cod protein**

### 4.3 Analytical methods to determine the composition of fava bean, fava bean products, beef and cod protein

A range of different methods; chemical, microbial and human *in vivo* have been used in the work related to this thesis, an overview is presented in **Table 1**.

**Table 1.** Overview of the methods used for studies related to this thesis.

	Method	Study
Chemical analyses		
Analyte		
Amino acids	LC-MS [152]	I, III
Ash	Combustion at 550°C	I
Dietary fiber, specific	GC [153]	I
Dietary fiber, total	GC, Uppsala method [154]	I, III, IV
Soluble/Insoluble fiber	GLC [155]	III
Fat, total	Extraction [156]	III
Fatty acids	GC-MS [157, 158]	III
Iron	Atomic absorption spectrometry	I, III, IV
Lectin	Hemagglutinating assay [159]	I
Oligosaccharides	HPIC	I
Phenolic content, total	Folin-Ciocalteu method [160]	I, III, IV
Phytate	HPIC [161]	I-IV
Protein, total	Total nitrogen, Dumas	I, III, IV
Saponin	Competitive ELISA [162]	I
Trypsin inhibition	Colorimetric method [163]	I
Vicine/Convicine	HPLC-DAD. Performed by LUKE, Finland [164]	I
Zinc	Atomic absorption spectrometry	I, III, IV
Salt content	ICP-MS, performed by Eurofins, Sweden	III
Microbial methods		
Genomic fingerprinting	rep-PCR [165]	II
Strain identification	MALDI-TOF MS	II
Probiotic potential	Tolerance to pH, bile, phenol, BSH activity	II
Safety	Antibiotic resistance	II
Methods related to mineral bioavailability		
Decreasing phytate content	Fermentation by LAB and yeast	II
Mineral bioavailability, estimated	Phy:Fe and Phy:Zn molar ratios	I, III, IV
Iron absorption <i>in vivo</i>	WBC and erythrocyte incorporation, radioiron isotopes	IV

### *4.3.1 Nutritional analyses*

#### *4.3.1.1 Protein*

Colorimetric methods, like Lowry [166], are commonly used for protein determination as they are fast and sensitive. However, the result can be affected by interfering substances present in the raw material [167]. Another common method for determination of the protein content in legumes is based on analysis of total nitrogen. Using a nitrogen-to-protein conversion factor, the total protein content can then be calculated. Total nitrogen methods have the disadvantage of being affected by non-protein nitrogen, but is not as sensitive to interfering substances as colorimetric methods. The Dumas method, total nitrogen through combustion, is faster compared to the total nitrogen method Kjeldahl which only measures organic nitrogen.

A commonly used nitrogen to protein factor is 6.25 across raw materials. This factor has been shown to overestimate protein content in legumes [168]. Instead, a conversion factor of 5.4, which correspond to an average for legume proteins [169], was used for plant-based samples in Study I, III and IV. For beef protein in Study III, a conversion factor of 5.48, and for cod protein a conversion factor of 5.58 were used [168]. For labelling purposes in the food industry, the Kjeldahl method is used in combination with a conversion factor of 6.25 across raw materials, which hence will both over and underestimate protein content depending on the sample. Important to note when comparing results between methods using the same nitrogen-to-protein conversion factor, the Dumas method has been reported to yield 1.4% higher protein content compared to the Kjeldahl method [170]. In Study I and III, the Dumas method was used, and in Study IV, the Kjeldahl method was used.

#### *4.3.1.2 Amino acids*

The quality of a protein is dependent on the amino acid profile [171]. Therefore the amino acid composition was analyzed in the materials used in study I and III. Prior to quantification by LC-MS, the samples were hydrolyzed under acidic conditions. Due to the use of harsh acidic hydrolysis, tryptophan could not be quantified since this amino acid was degraded during the hydrolysis [172]. The content of tryptophan in legumes is low (approximately 0.8% of total protein content) and was therefore considered to constitute a minor fraction [173].

#### *4.3.1.3 Iron and Zinc*

Atomic absorption spectrometry was used for determination of iron and zinc content in studies I, III and IV. Prior to analysis the samples were degraded using microwave digestion in acidic conditions, as described by Fredrikson et al [174].

#### *4.3.1.4 Ash*

The ash content was determined for fava bean cultivars in paper I. Ash content was determined by combustion at 550°C. By ashing, the organic content is removed which leaves inorganic minerals.

#### *4.3.1.5 Dietary fiber*

In study I and IV, total dietary fiber was analyzed according to the Uppsala method [154]. In addition, soluble, insoluble and specific fiber components were analyzed according to the method described by Andersson et al [175]. Analysis of fava bean samples in Study I was performed at the Swedish University of Agricultural Sciences in Uppsala. Analyses for Study IV was performed using a modified version of the Uppsala method [155] at Aarhus University, Denmark.

#### *4.3.1.6 Total fat*

Extraction of fat was done according to Bligh et al [156], with slight modifications. In duplicates, 0.2 g of freeze-dried and ground meat substitute was added to 5 mL of chloroform:methanol (2:1) (Sigma Aldrich and Fischer Scientific) in a glass vial and incubated at room temperature in a rotating shaker (Heidolph Reax 2; Heidolph Instruments GmbH) for 30 minutes and then sonicated (Branson ultrasonic bath model 8510-DTH) in a water bath for 5 minutes. The incubation and sonication steps were repeated twice. To stop the reaction, 1.5 mL of Milli-Q was added to the vial and centrifuged for 2 minutes at 4000 x g. The chloroform layer was collected into a new glass vial and 4 mL pure chloroform was added into the previously centrifuged tube to collect any remaining fat. After vortexing and centrifugation, at 4000 x g, chloroform was again removed and pooled. Chloroform:fat solution was then evaporated under nitrogen until stable weight of the vial.

#### *4.3.1.7 Fatty acid composition*

Fatty acids in meat substitutes of Study IV were analyzed by direct trans-esterification of freeze fried samples. Fatty acid methyl esters (FAME) were quantified using GC-MS. Analysis was done according to the method described by Lepage et al [157] and modified by Cavonius et al [158].

#### *4.3.1.8 Determination of salt content*

As meat substitutes has been receiving critique for having high salt content, we analyzed the content of salt (NaCl) in meat substitutes of Study IV. An accredited method based on pressure digestion, SS-EN 13805:2014 [176], was used and carried out by Eurofins, Lidköping, Sweden

### *3.1.1 Analyses of antinutritional compounds*

#### *4.3.2.1 Analysis of phytate content*

Several different quantification methods have been developed aimed at determining the level of phytate, with a wide range of sensitivity and selectivity between inositol phosphates. Titration methods as well as spectrophotometric methods were originally developed as these are simple to use. These early methods are not specific and have low sensitivity for phytate (InsP6) as the methods cannot distinguish between phytate and its dephosphorylated degradation products (InsP5-InsP1), nor inorganic phosphates that form insoluble precipitates with  $\text{Fe}^{3+}$  [177]. Using nonspecific analytical methods may therefore be misleading. When analyzing phytate for mineral bioavailability purposes, it is important to analyze the InsP<sub>n</sub> compounds separately. In processed foods, InsP3-InsP5 contribute to the inhibition of mineral absorption, while InsP6 is the most potent due to the largest number of phosphor groups [178-180]. Due to large differences between specificity and sensitivity, it is of high importance to take analytical methods into consideration when comparing analyzed phytate contents between studies [181].

The high pressure ionic chromatogram (HPIC) method used in the studies related to this thesis, the method by Carlsson et al [161], developed in our laboratory, is highly sensitive with a limit of detection of 0.5 µg. This method offers high specificity and large

advantages over colorimetric or titration methods as HPIC separates different  $\text{InsP}_n$  compounds, and is not affected by inorganic phosphates. In the studies included in this thesis, only  $\text{Insp}_6$  was quantified as this had some advantages over a complete investigation of  $\text{InsP}_n$  compounds. For example,  $\text{Insp}_6$ -chromatograms have a runtime of seven minutes, compared with 30 minutes for a chromatogram including all  $\text{InsP}_n$  compounds. While a more detailed analysis using an isomer gradient is more time consuming, it is more sophisticated and will give a complete overview of phytate and its degradation products. However, in samples that has not undergone phytate degrading processes,  $\text{Inp}_6$  will be the primary inositol phosphate as this is the main storage form of phosphorus and inositol in plants [182]. In Study I where only raw fava bean varieties were analyzed, only a very low content of phytate degradation products were expected. In Study II, where fermentation was used to degrade phytate, only  $\text{Insp}_6$  was analyzed as this reveal if a strain has the ability to reduce phytate and if it is relevant for further investigation. In Study III and IV, extracted and texturized legume protein products were analyzed. As  $\text{Insp}_6$  is known to accumulate in the protein fraction of extracted plant proteins, a high content of  $\text{Insp}_6$  was expected and hence the relevance of other  $\text{InsP}_n$  compounds was not considered as significant.

#### *4.3.2.2 Estimation of relative iron and zinc bioavailability*

A common way to estimate the theoretical bioavailability of non heme iron and zinc from a plant-based food matrix is to calculate the molar ratio of phytate ( $\text{Insp}_6$ ) to mineral. Since the inhibition of phytate on both non-heme iron and zinc absorption is dose dependent in human subjects, a low phytate:mineral (phy:mineral) molar ratio correspond to a high theoretical bioavailability [136, 183, 184]. According to the European Food Safety Authority (EFSA), a phy:Zn molar ratio below five correspond to a high absorption efficiency, 5-15 moderate and above 15 is considered to reflect a low bioavailability [185]. For iron, FAO/INFOODS/IZINCG recommends using the phy:Fe molar ratios presented as dietary reference values suggested by Hurrell and Egli [151, 186]. According to these recommendations, the phy:Fe molar ratio should be below 1, or preferably below 0.4, to significantly improve non heme iron absorption in plant-based meals with no iron absorption stimulating factors. In meals containing both meat factor and ascorbic acid, the phy:Fe molar ratio should not exceed 6. A molecular mass for phytate of 660.3 g/mol was used for the

calculations. In this thesis, ‘estimated bioavailability’ refers to the calculated phytate:mineral molar ratio.

#### *4.3.2.3 Vicine and Convicine Analysis*

In study I, fava bean cultivars were analyzed for their content of vicine and convicine by the Natural Resources Institute of Finland (LUKE), according to the method of Gutierrez et al [164].

#### *4.3.2.4 Determination of lectin*

The most common method for determination of lectins is a semi-quantitative hemagglutinating assay, where the level of lectins is calculated using the highest dilution of extract where agglutination of erythrocytes can be detected visually [159, 187]. Despite being widely used, hemagglutination assays have several major drawbacks such as variations in results depending on blood cell source, blood type used and lab conditions. As an alternative, ELISA-systems using immobilized glycoproteins has been developed [188, 189]. Despite the difficulties related to the semi-quantitative hemagglutination assay, it is still the most common method for lectin determination and was used in study I. Direct comparison of results between different studies and labs should therefore be made with consideration of the limitations.

#### *4.3.2.5 Analysis of Saponin*

Saponin was quantified in Study I using a competitive ELISA with a monoclonal antibody that recognizes unconjugated soyasaponin I, developed by Frøkiær et al [190].

#### *4.3.2.6 Determination of trypsin inhibition*

Determination of trypsin inhibition was determined in Study I by the colorimetric method developed by Verdenelli et al [191].

#### *4.3.2.7 Analysis of the Total Phenolic Content*

Total phenolic content (TPC) was determined in samples of fava bean cultivars in Study I, and plant-based food products in Study III. Determination was made by the Folin-Ciocalteu method based on the technique of Howard et al [160]. Analyzing the total phenolic content, rather than specific phenolic compounds, can be used as a basic rapid screening. To investigate negative effects on iron-absorption by polyphenols, further analysis of specific iron-binding polyphenols is required.

#### *4.3.2.8 Determination of Oligosaccharides*

In study I, oligosaccharides raffinose, verbascose and stachyose were analyzed by HPIC. A detailed description of the method can be found in Paper I.

### *4.3.3 Microbiological methods*

#### *4.3.3.1 Identification of isolated strains*

In Study II, microbial colonies were isolated from legume samples collected on Öland, Sweden. Collection of legumes was made from fields and from stored seeds, using sterile gloves. Initial isolation was made using Man, Rogosa and Sharpe (MRS) broth and MRS plates at anaerobic conditions. Colonies that were Gram-positive, oxidase-negative and catalase-negative were selected for identification. Genomic DNA was extracted from a total of 116 isolates, according to the method described by Sjöberg et al [192]. To reduce the risk of duplicate isolates, repetitive polymerase chain reaction (rep-PCR) was used to assess strain diversity, by the method according to Noriega et al [193]. Rep-PCR fingerprints were analyzed using BioNumerics (Applied Maths NV, Sint-Martens-Latem, Belgium). A representative isolate was selected randomly from each cluster of strains belonging to the same genotype. Representative strains were analyzed twice with matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) for identification.



#### 4.3.3.2 Phenotypic tests related to probiotic potential

For a strain to be considered as a candidate probiotic and relevant for further investigation, a number of prerequisites needs to be fulfilled. In Study II, the ability to survive harsh conditions in the gastro-intestinal tract (GIT) was studied, including tolerance to bile (using the method described by Gilliland and Walker [194]), low pH [195] and phenol [196]. The ability to produce BSH enzyme has often been included as a criterion for selection of probiotic strains, and was investigated in strains isolated in Study II [197]. The ability to detoxify bile salts by producing the enzyme bile salt hydrolase was measured in strains qualitatively according to Cook et al [198]. Apart from indicating survival of the toxicity from bile salts, BSH activity also indicate de-conjugation of these salts which might help with intestinal colonization of cells and in the reduction of blood cholesterol level in the host [199].

#### 4.3.3.3 Mono and co-fermentation of fava bean drink for decreased phytate content

Bacterial strains of *P. pensosaceus* from study I, isolated from Swedish legumes, were selected for mono fermentation of a fava bean drink. As fava bean contain both minerals and phytate, it is an interesting substrate to study for potential phytate degradation. A *P. pensosaceus* strain from the mono fermentation, which were able to ferment the fava bean drink with no added nutrients, was selected to co-ferment the substrate with the yeast strain *Pichia kudriavzevii* TY1322, a strain known to express phytase [150]. *Pichia kudriavzevii* TY1322 was also inoculated for mono fermentation in order to examine the combined effect compared to single strains. Negative controls were samples without inoculation, treated the same way as other samples. Incubation was done aerobic in a rotary shaker at 37°C.

#### 4.3.3.4 Extraction of beef and cod protein

Extraction of beef and cod protein used in test meals of Study IV was done using the pH-shift method according to Abdollahi et al [200], with minor modifications. In order to scale up the production from lab scale, phases were separated using a Russell Finex vibrating sieve which enabled higher capacity compared to lab centrifuges.

#### *4.4. Measurement of iron absorption in humans*

Absorption of dietary iron has been investigated by a number of different methods such as hemoglobin incorporation of stable or unstable isotopes, fecal monitoring or whole body counting able to detect  $^{59}\text{Fe}$  [201]. Whole body counting (WBC) in combination with hemoglobin incorporation using double radioisotopes is considered the gold standard. Human studies using radioactive iron isotopes have the advantage of requiring a very small, essentially mass free, amount of iron added to test meals. In comparison, stable iron isotopes have to be administered in larger amounts, which can alter the ratio of iron to phytate, and affect physiological conditions during the trial [202]. As stable isotopes exist in nature, corrections must be made for background levels when used as tracers in absorption studies [203]. Further on, the gamma-isotope  $^{59}\text{Fe}$  can be detected by a WBC, which enables absolute measurement of the total  $^{59}\text{Fe}$  activity in subjects and not only in erythrocytes as is the case for stable isotopes. In combination with WBC, hemoglobin incorporation enables the use of double isotopes  $^{55}\text{Fe}$  and  $^{59}\text{Fe}$  as the ratio of the two isotopes can be measured in a liquid scintillator. Hemoglobin incorporation can also be used without WBC, the percentage of iron absorption is then calculated from estimates of blood volume and an assumed erythrocyte incorporation of absorbed iron to 80% [204]. The actual individual percentage of absorbed radioiron that is incorporated into erythrocytes as well as total blood volume of subjects are unknown and sources of error [205]. Due to large individual variations, correction relative to a reference dose or serum ferritin levels is standard to be able to compare absorption between subjects and between studies [206, 207]. In study IV, absolute absorption of  $^{59}\text{Fe}$  was measured by WBC. The absorption of  $^{55}\text{Fe}$  was calculated from absolute WBC data, using relative absorption of  $^{59}\text{Fe}$  and  $^{55}\text{Fe}$  in erythrocytes. Adjustment to a reference dose of ferrous ascorbate was applied relative to a 40% absorption level, corresponding to borderline iron-deficient subjects. Even though this model has limitations, and has received critique due to variances caused by individual iron status [208], it has been widely used and was considered most useful for comparison between studies.

#### *4.5 Statistical analyses*

Results of normally distributed data are presented as arithmetic mean values, while non-normally distributed data are presented as median values. Statistical analyses were performed using Microsoft Excel 2002 (Microsoft, Albuquerque, New Mexico, USA) and the IBM SPSS

Statistics ver. 27.0 (IBM, New York, NY, USA). To determine if there was a significant difference between two groups, t-test was used for normally distributed data, and Mann-Whitney U test for non-normally distributed data. Differences were denoted significant when  $p < 0.05$ . Specific information is found in respective papers I-IV.

## 5. RESULTS AND DISCUSSION

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The extensive chemical analyses of fava bean cultivars and meat substitutes reveal large variations in the content of nutrients and antinutrients. Two of the analyzed cultivars of fava bean had promising low content of phytate. Largest variation in nutrients and antinutrients were found among meat substitutes. A majority of the meat substitutes had high contents of salt and saturated fat, and a low estimated bioavailability of iron and zinc. Most of the products had high fiber contents (5.6-21.5% of dry matter). Data on meat substitutes were sorted and analyzed according to the main source of protein, with extracted soy protein as the most abundant followed by extracted pea protein. Lipid profiles were in contrast sorted and analyzed according to added fat, since the additive effect of fat overshadowed all other groupings.

In Study II, bacterial strains were isolated from Swedish legumes and investigated for their potential probiotic potential as well as phytate degrading properties in co-fermentation with a phytase-secreting yeast strain. A total of 25 unique strains were isolated and identified. A strain of *Pediococcus pentosaceus* was able to markedly improve the phytate degradation in co-fermentation with the yeast strain. The strain combination can have industrial applications to develop innovative new plant-based products with improved nutritional quality.

Finally, in Study IV, extracted and texturized fava bean protein was investigated in human clinical single meal trials. The clinical setup was comprised of two separate studies where the non-heme iron absorption of texturized fava bean protein was compared with 1: beef protein, and 2: cod protein. Healthy women of fertile ages were used as study population, with separate participants in the two trials. Results revealed a significantly lower absorption of non-heme iron from texturized fava bean compared with the beef and cod protein meals.

As the nutritional composition and quality is largely dependent on the processing methods and formulation of the final product, meat substitutes can be substantially improved with the addition of nutritional competence to product development.

## 5.1 Nutrient and antinutrient profiles of fava bean cultivars, meat substitutes and test meals

### 5.1.1 Protein content

In study I, protein content varied among cultivars and was determined to be from 26% in cultivar Alexa up to 33% in the high-protein cultivar Gloria (**Table 2**). Previous studies on fava bean have determined protein content to be in the range of 22%-38%, which confirm that there are large variances between cultivars [187, 209, 210]. In Study III, protein content varied between 5.5g/100g to 23.8g/100g product. While large variances were seen among samples, median protein content was similar between categories of meat substitutes. No significant difference was found between categories, sorted and analyzed based on protein source. The digestibility of protein exposed to different protein extraction methods and extrusion should however be investigated further.

**Table 2.** Protein content of fava bean cultivars of Study I sorted and analyzed according to flower type, and meat substitutes of Study III, sorted and analyzed on the basis of main protein source

	Sample group	n	Protein content
Study I	<i>Fava bean cultivar type:</i> Color flowered	10	24.2 (23.1-25.0)
	White flowered	5	25.0 (24.4-27.7)
Study IV	<i>Meat substitutes based on:</i> Soy and wheat protein	8	13.0 (11.4-5.4)
	Whole bean	4	8.7 (7.0-11.4)
	Soy protein	9	12.5 (11.1-14.3)
	Pea protein	12	9.5 (6.9-12.9)
	Mycoprotein	5	13.3 (10.4-14.4)
	Tempeh	1	6.7 ± 10%
	Other	3	14.0 (11.9-23.8)

Protein content is presented as median g/100g DW for data from Study I, and g/100g product for data in Study III. Apart from tempeh, results from Study III are presented as median (25<sup>th</sup> percentile – 75<sup>th</sup> percentile). Tempeh is presented as average ± measurement uncertainty as reported by Eurofins laboratory

### *5.1.2 Amino acid composition*

The amino acid patterns were similar between fava bean cultivars in Study I. As expected, the fava bean cultivars had a high content of leucine, lysine, aspartic acid, arginine and glutamic acid. Thus, fava bean can be considered complementary to cereal grains which in general are low in lysine and high in Sulphur-containing amino acids [211].

Results from meat substitutes analyzed in study IV showed that amino acid composition in products based on protein extracts are more complex to predict compared with what is known for the raw material. Content of the essential amino acid methionine was low in products based on extracted legume protein and whole legumes. Whole cereals contain a high amount of methionine, which is the reason for cereals and legumes to be considered complementary protein sources [211]. However, products containing extracted cereal protein also had a low content of methionine according to findings in Study III which suggest that extracted and extruded cereal protein is not complementary. Previous studies have shown that extrusion cooking affects the amino acid composition, as the level of certain amino acids decrease during extrusion. The magnitude of the effect and affected amino acids are dependent on the extrusion conditions [129]. The processing methods and settings used for production of meat substitutes in Study III were not known in detail, but it is clear that choice of crop, cultivar, extraction method as well as following processing methods such as extrusion cooking, will have an effect on the amino acid composition of the product.

### *5.1.3 Dietary fiber*

The total content of dietary fiber in fava bean varieties analyzed in Study I varied between 11.4% in cultivar Daisy to 16.7% cultivar Stella. As expected, the insoluble dietary fiber fraction was predominant and in the range of 10.7% in cultivar Daisy to 16.0% in cultivar Stella. Soluble dietary fiber content varied between 0.55% in Tiffany and Banquise to 1.06% in FanFare. Total dietary fiber content was slightly higher in white flowered cultivars (**Table 3**). In Study III, total dietary fiber varied between 4.4% in Wheat fish sticks (category “Other”), to 21.5% in Tempeh burger. Insoluble fiber fraction varied between 4.1% in Wheat and pea nuggets (category “Other”) to 15.4% in Tempeh burger. Tempeh burger also had the highest content of soluble fiber with 6.1%; lowest soluble fiber content was found in Mycoprotein schnitzel (0.7%). In the test meals of Study IV, the fava bean meal had the highest content of total, soluble and insoluble fiber compared with the beef and cod protein meals.

**Table 3.** Overview of total dietary fiber and the soluble and insoluble fractions of fava bean cultivars of Study I, meat substitutes of Study III and test meals of Study IV

	Sample	n	Total DF	Soluble DF	Insoluble DF
Study I	Fava bean cultivars, colour flowered	10	13.9 (12.8-15.2)	0.7 (0.6-0.7)	13.1 (12.2-14.4)
	Fava bean cultivars, white flowered	5	15.1 (13.2-15.8)	0.6 (0.6-0.8)	14.3 (12.6-15.0)
Study III	Meat analogues based on:				
	Soy and wheat protein extract	8	10.9 (8.0-13.9)	3.3 (4.5-4.9)	7.7 (5.9-10.0)
	Whole bean	4	11.4 (9.2-13.2)	3.6 (2.1-5.1)	6.8 (6.6-10.0)
	Soy protein extract	9	10.5 (9.6-13.3)	4.3 (3.3-4.9)	6.7 (6.0-8.2)
	Pea protein extract	12	8.0 (7.1-9.7)	2.7 (2.1-3.3)	5.3 (4.3-6.6)
	Mycoprotein	5	9.9 (6.8-17.8)	1.3 (1.0-3.4)	7.4 (5.8-16)
	Tempeh	1	21.5	6.1	15.4
	Cheese	2	6.7 (6.2-7.3)	2.2 (1.8-2.5)	4.6 (4.4-4.8)
	Other	3	5.6 (4.4-8.8)	1.4 (0.5-4.3)	4.1 (3.9-4.5)
Study IV	Test meal based on:				
	Fava bean protein	1	10.3	4.6	5.8
	Beef protein	1	3.1	0.9	2.2
	Cod protein	1	3.5	1	2.5

Dietary fiber and fiber fractions are presented as percentage of total dry weight  
Results are presented as median (25th-75th percentile). Data on tempeh and test meals are presented as average value from duplicates  
Abbreviations: DF: dietary fiber

From the chemical analyses of Study I and III, we could conclude that fava bean cultivars and meat substitutes are good sources of dietary fiber which may reduce the postprandial blood sugar and insulin responses, gastrointestinal transit time [212, 213] and reduce the low-density lipoprotein (LDL) cholesterol [214]. As expected, tempeh had the highest content of total, soluble and insoluble dietary fiber. Dietary fiber might have beneficial effects in protecting against certain forms of cancer, reducing blood pressure and exerting an anti-inflammatory effect in the digestive tract [215, 216]. Certain dietary fibers have also been attributed with a mineral-binding capacity, which might lead to inhibitory effects on mineral absorption [217-219]. However, human studies investigating a potential effect of dietary fiber on mineral absorption are scarce, but have failed to confirm a negative effect of insoluble and soluble fibers on mineral absorption [220, 221].

#### *5.1.4 Total fat and fatty acid composition*

Results on fatty acid analysis of meat substitutes was sorted and analyzed according to which fat that had been added to the product, as stated on the package, and not based on protein source as the added fat affected the results to a larger extent compared with protein source (**Table 4**). As was expected, products that had been added with coconut, shea or palm oil had a higher content of saturated fat compared to products added with only rapeseed oil and sunflower oil. Products added with only sunflower oil had an elevated content of saturated fats compared to products added with a blend of sunflower and rapeseed oil, only rapeseed oil and products containing no added fat. In this case, the saturated fatty acids originated from the protein source, which was cheese. Highest content of the polyunsaturated linoleic acid (LA) was found as expected in products added with sunflower oil, this was also the product category with the least amount of omega 3 (n-3) fatty acids. Results from Study III show that choices made during formulation of meat substitutes will significantly affect the fatty acid composition. Nutritional considerations during recipe development are, as assumed self-explanatory, crucial for the nutritional composition and health implications of a products.



**Table 4.** Fatty acid composition of meat substitutes analyzed in Study IV

Added fat	Total fat	Saturated FA	Mono-unsaturated FA	n-3 FA*	essential FA**
No fat (n=3)	2.4 (2.2-2.7)	0.37(0.29-0.37)	0.4 (0.31-0.42)	0.07 (0.06-0.11)	1.0 (0.93-1.32)
C, S or P and rapeseed oil (n=5)	16.3 (10.5-17.5)	2.03(1.73-3.22)	5.76 (3.49-6.76)	0.59 (0.28-0.95)	2.82 (1.52-4.58)
Rapeseed oil (n=22)	12.8 (9.1-16.2)	0.8(0.69-0.99)	5.37(4.06-7.61)	0.60 (0.47-0.88)	3.15 (2.23-4.07)
SF and rapeseed oil (n=6)	10.9 (7.2-12.1)	0.9 (0.51-2.14)	3.99 (2.69-4.69)	0.42 (0.28-0.51)	2.16(1.63-2.89)
SF oil (n=7)	13.2 (11.0-14.2)	1.13 (1.04-1.37)	3.15 (2.44-3.21)	0.0 (0.00-0.04)	5.89 (5.65-9.64)
C and SF oil (n=1)	13.4	3.23	2.69	0.02	4.31

Results are presented as median value (25<sup>th</sup>-75<sup>th</sup> percentile), g/100g product

\*omega 3 (n-3) fatty acids consist of alpha linolenic acid (ALA) as no EPA or DHA were present in the samples

\*\*Essential fat were ALA and linoleic acid (LA)

Abbreviations: FA: fatty acids; C, coconut oil; S, shea butter; P, palm oil; SF, sunflower oil

#### *5.1.5 Phytate, iron and zinc content, and estimated bioavailability in fava bean cultivars, fava bean drink, meat substitutes and fava bean test meal*

Fava bean cultivars in Study I had in general a high phytate content (**Table 5**). Since phytate is known to interfere substantially with the absorption of minerals [136, 184], the high phytate levels can thus be assumed to affect the cultivars included here in the same way. Two of the cultivars (Sunrise and Lynx) did however contain a low content of phytate in combination with high iron contents, which resulted in phy:Fe molar ratios below 6 which is suggested as the maximum molar ratio where the inhibitory effect of phytate can be counteracted with simultaneous addition of both vitamin C and the meat factor [151]. Cultivar Sunrise had an exceptionally high iron content and low content of phytate which is reflected in the low phy:Fe molar ratio of 0.4, which can be considered a high bioavailability of iron even without stimulating factors [151]. In addition, cultivar Sunrise had a high content of zinc and a phy:Zn molar ratio of 2.1 which is within the range of good bioavailability according to guidelines from WHO and EFSA [186]. These findings suggest that it might be possible to make selection of varieties with high iron and low phytate contents, thus likely higher iron and zinc bioavailability, through screening.

Very large variances in phytate, iron and zinc contents were found in meat substitutes of Study III. Neither of the products of Study III were found to be good sources of iron, due to a low iron content and/or a high phytate content. Out of the 44 products analyzed, 26 (59%) had an iron content  $\geq 2.1$  mg/100g, which is the lower limit for a nutritional claim on iron according to EU regulations [222]. Only three of the products with an iron content of  $\geq 2.1$  mg/100g had a phy:Fe molar ratio of  $\leq 6$  (Pea burger 2, Pea sausage 3 and Soy and wheat balls 3), and neither of the products were below the limits of 1 or 0.4. Results from Study III indicate that meat substitutes analyzed had very low bioavailability of iron, and that it is difficult to meet the iron needs on a diet mainly consisting of these products as iron sources. In total, 6 products had a nutritional claim on iron (Cheese patty 1, Chickpea falafel 2, Oat bites, Soy wheat bacon, Soy wheat balls 3, Soy wheat schnitzel and Soy balls). The Cheese patty 1 had an iron content below 2.1mg/100g (1.8mg/100g). The phy:Fe molar ratios of the meat substitutes with a nutritional claim on iron, and a simultaneous iron content of  $\geq 2.1$  mg/100g were 14.2 (Chickpea falafel 2), 9.2 (Oat bites), 6.1 (Soy wheat bacon), 3.9 (Soy wheat balls 3), 6.6 (Soy wheat schnitzel) and 12.1 (Soy balls). Hence, only one of the products, Soy wheat balls, was below the suggested maximum phy:Fe molar ratio where it is possible to counteract the negative effects of phytate on iron absorption with stimulating factors.

**Table 5.** Phytate, iron and zinc content, and molar ratios of Phy:Fe and Phy:Zn

Study I	Sample type	Sample	Phytate	Fe	Zn	$\Delta$ Phy:Fe	$\Delta$ Phy:Zn
	Mature fava bean	Lynx	313 $\pm$ 6	7.0 $\pm$ 0.18	1.4 $\pm$ 0.02	3.8	22.1
		Sunrise	112 $\pm$ 4.2	21.3 $\pm$ 0.29	5.2 $\pm$ 0.06	0.4	2.1
		Cultivars not presented individually, combined (n=13)	612-1281	1.8-5.5	0.9-3.8	12.9-22.2	26.0-62.7
Study II	Fermented fava bean drink	Starting level	39.6 $\pm$ 1.2				
		TY1322	29.5				
		LAB 77	40.1				
		Mixed fermentation	9.1				
Study III	Meat substitutes	Pea burger 2	80 $\pm$ 2.8	2.7 $\pm$ 0.06	1.0 $\pm$ 0.03	2.5	4.6
		Pea sausage 3	126 $\pm$ 1	3.1 $\pm$ 0.08	1.7 $\pm$ 0.02	3.5	7.2
		Soy and wheat balls 3*	178 $\pm$ 3.7	3.8 $\pm$ 0.07	1.0 $\pm$ 0.03	3.9	18.3
		Mycoprotein bites	<0.01	0.4 $\pm$ 0	8.7 $\pm$ 0.09	0.7	0.0
		Mycoprotein schnitzel	<0.01	0.8 $\pm$ 0.02	4.5 $\pm$ 0.21	1.8	0.4
		Mycoprotein burger	<0.01	0.7 $\pm$ 0.01	4.2 $\pm$ 0.04	0.6	0.1
		Mycoprotein mince	<0.01	0.5 $\pm$ 0.02	7.2 $\pm$ 0	0.6	0.0
		Mycoprotein filets	<0.01	0.5 $\pm$ 0	6.7 $\pm$ 0.04	0.5	0.0
		Cheese patty 1*	21 $\pm$ 0.6	1.8 $\pm$ 0.03	1.1 $\pm$ 0.02	1.0	1.9 $\pm$ 0.09
		Oat bites*	255 $\pm$ 6.4	2.5 $\pm$ 0.04	1.3 $\pm$ 0.01	9.2 $\pm$ 0.39	20.4 $\pm$ 0.43
		Soy and wheat schnitzel*	359 $\pm$ 12.5	4.6 $\pm$ 0.06	1.2 $\pm$ 0.02	6.6 $\pm$ 0.15	1.42 $\pm$ 4.6
		Soy and wheat bacon*	336 $\pm$ 6.4	4.7 $\pm$ 0.02	1.0 $\pm$ 0	6.1 $\pm$ 0.09	33.1 $\pm$ 0.6
		Soy balls*	338 $\pm$ 3.1	2.4 $\pm$ 0.33	1.3 $\pm$ 0.01	12.1 $\pm$ 1.79	44.6 $\pm$ 0.39
		Chickpea falafel*	736 $\pm$ 32.2	4.4 $\pm$ 0.02	1.7 $\pm$ 0.05	14.2 $\pm$ 0.55	42 $\pm$ 0.71
		Meat substitutes not presented individually, combined (n=30)	9-1151	1.0-4.7	0.8-2.2	1.5-43	(0.6-67)
Study IV	Protein extract	Texturized fava bean protein	2722	5.7	3.7	41.7	
	Test meal	Texturized fava bean protein meal	1692	5.1	3.3	38.7	

Data is presented as average of duplicates  $\pm$  standard deviation

Phytate and mineral content is presented as mg/100g DW for study I, II and texturized fava protein in Study III, mg/test meal in Study III and mg/100g product in Study IV

From study I, only cultivars with a Phy:Fe molar ratio below 6, or Phy:Zn below 15 is presented

From study IV, samples with an iron content above 2.1 mg/100g and a Phy:Fe molar ratio below 6 is presented individually, as well as products with a nutritional claim on iron

\*Product had a nutritional claim on iron

Zinc content of meat substitutes were consistently low, apart from products based on mycoprotein. Since these products did not contain any phytate, they can be expected to have a high bioavailability of zinc. Since zinc is one of the risk nutrients identified in a plant-based diet [15], this is an important finding and producers should be encouraged to make a nutrition claim on zinc. As the nutritional composition of mycoprotein is dependent on which species is used, as well as the composition of the substrate and conditions during biomass growth [223], nutritional knowledge should be incorporated in the development of mycoprotein products. Mycoprotein products in Study III had a very low content of iron, but is an interesting raw material to further improve since it does not contain any known inhibitors of mineral absorption.

#### *5.1.6 Oligosaccharides*

The concentration ranges of Raffinose Family Oligosaccharides (RFOs) in fava bean cultivars in Study I were 1.1–3.9 g/kg DW for raffinose, 4.4–13.7 for stachyose, and 8–15 g/kg DW for verbascose. Landry et al reported a raffinose content of fava bean varieties in the range of 2.7–13.0 g/kg, a stachyose content of 9.0–25.0 g/kg DW, and verbascose content of 6.7–50.3 g/kg DW [224]. In Study III, as other previously reported sources, the variability in concentration of RFOs has been shown to be considerably high among fava bean cultivars. RFOs are known to cause post-prandial flatulence and discomfort and might trigger gastrointestinal symptoms sensitive individuals. High levels of RFOs can therefore be considered a problem in food products [225]. Yet, RFOs are prebiotics that may provide positive health effects for the consumer, such as increasing the bifidobacterial population in the gut, improving the immune system response, and decreasing risk factors associated with obesity, metabolic syndrome and colon cancer [226-228]. A low level of RFOs can help to increase the acceptability of legumes, such as fava bean [229].

#### *5.1.7 Antinutrients*

Apart from phytate and phenolic compounds, which inhibit mineral absorption, lectin; saponin; vicine/convicine and trypsin inhibition was investigated in Study I. Since these compounds are inactivated by heat, it was assumed that processing methods used for production of meat substitutes of Study III had removed, or significantly reduced, these compounds. Therefore, they were not included for analysis in Study III.

The content and activity of these antinutrients were coherently low for some of the cultivars investigated in Study I. These were foremost cultivars Emilia and Tiffany, which were in the lowest range for each of these antinutrients. The material analyzed was cultivated in Sweden during the summer season of 2018, which was exceptionally dry and affected by drought. The results of Study I needs to be verified for other growing seasons as well as cultivation with other soil properties, since ecological and agronomical factors has been reported to affect the content of antinutrients in plants [230-232].

#### *5.1.8 Total phenolic content*

In white flowered cultivars in Study I, the total phenolic content (TPC) was low and can be assumed as not likely to interfere with iron absorption (**Table 6**). The TPC content in color flowered fava bean cultivars and meat substitutes indicate that iron-binding polyphenols might be present, but it is not possible to draw any conclusions on potential effects. As we analyzed the TPC and not specific phenolic compounds, further studies are needed to identify iron-binding polyphenols in color flowered fava bean varieties and meat substitutes. Specifically, phenolic compounds with galloyl and catechol functional groups has been ascribed with iron-binding properties [233]. The influence of plant protein extraction and extrusion on iron-binding polyphenols is also unknown and should be investigated as these methods are becoming increasingly popular in the production of meat substitutes.

**Table 6.** Total phenolic content in fava bean cultivars, separated into color flowered and white flowered cultivars, the fava bean test meal and meat substitutes, sorted by protein source

	Sample	n	TPC
Study I*	Fava bean cultivars, color flowered	10	2.8 $\pm$ 0.06 - 5.0 $\pm$ 0.06
	Fava bean cultivars, white flowered	5	1.4 $\pm$ 0.01 - 2.3 $\pm$ 0.04
<i>Products based on:</i>			
Study III**	Soy and wheat protein extract	8	10.6 (9.6-15.4)
	Whole bean	4	19.3 (7.0-37.7)
	Soy protein extract	9	11.7 (9.4-26.5)
	Pea protein extract	12	10.2 (9.4-26.5)
	Mycoprotein	5	8.5 (7.4-9.8)
	Tempeh	1	21.2 $\pm$ 0.003
Study IV***	Fava bean protein test meal	1	13.7 $\pm$ 0.003

Total phenolic content (TPC) is presented as the average mg gallic acid equivalents (mg GAE/g)  $\pm$  standard deviation for study I, IV and tempeh in study III.

Apart from tempeh, results from Study III are presented as median (25<sup>th</sup> percentile – 75<sup>th</sup> percentile)

\*In Study I, results are in dry weight, \*\*\*In Study III, results are presented as mg GAE/100g product,

\*\*In Study IV, result is for the total study meal

#### 5.1.9 General compositional aspects for a healthy green protein shift

Results from Study I and III show large variances in the composition of fava bean cultivars and meat substitutes. Among cultivars, two had a markedly lower content of phytate (Lynx and Sunrise). In addition, Sunrise had a high content of both iron and zinc, which makes Lynx and especially Sunrise interesting cultivars for food production. Fava bean cultivars had a high content of dietary fiber and in general a high protein content. Integrating nutrition into plant breeding programs aimed at optimizing the nutritional yield, rather than only optimizing the yield of protein or tons, can positively affect the nutritional gain from legume consumption.

Among meat alternatives, a majority had a high salt content and many alternatives contained high levels of saturated fats. Mycoprotein products and tempeh differentiated from other products in that they had low contents of salt, saturated fats and phytate. Tempeh, although

the sample size was limited to only one product, had the highest content of total, soluble and insoluble dietary fiber. This was expected as tempeh is produced from whole, preboiled and fermented legumes and does not contain any extracted ingredients. Based on these results, tempeh is a promising meat substitute with a composition that can be classified as “healthy”. Mycoprotein products had significantly higher zinc content compared with other products, and no known inhibitor or mineral absorption. Products from this category also contained dietary fiber, which together makes mycoprotein an interesting raw material for further development of novel food products. While all meat substitutes analyzed contained dietary fiber beneficial for health, and the content of added salt and fats can be greatly improved by re-formulation, there are some question marks regarding the bioavailability of nutrients from extracted protein products. Improvement of the nutritional quality of meat substitutes, foremost those based on protein extracts, should be a prioritized step of the green protein shift.

## 5.2 Microbiological results

### 5.2.1 Strain identification

In Study II, 116 isolates of putative LAB were obtained from 23 legume samples collected on Öland, Sweden. The isolates fulfilled criteria used to select LAB and closely related lactate-producing bacteria. The genomic fingerprinting method rep-PCR was used to cluster isolates into groups based on the fingerprint patterns. Fingerprints that showed >90% similarity were assigned to the same genotype and were considered a cluster, as suggested by Maluping et al. [234]. One strain was randomly selected from each cluster as a representative for the group, which resulted in 25 unique strains. Each of these 25 strains was analyzed by MALDI-TOF MS, which identified the strains as *Enterococcus hirae*, *Enterococcus faecium*, *Enterococcus mundtii*, *Bacillus coagulans* and *Pediococcus pentosaceus*.

### 5.2.2 Fermentation as a method to reduce the phytate content in fava bean drink

Strains from the LAB *P. pentosaceus* were able to ferment a fava bean drink without any added nutrients, suggesting *P. pentosaceus* strains to be suitable for further investigation and use. The strains did not have the ability to degrade phytate, which is in line with previous reports on LAB fermentation of legume drinks [145]. In a mixed fermentation, combining *P. pentosaceus* with the phytase producing yeast strain *Pichia kudriavzevii* TY1322, the degradation of phytate was more efficient compared with mono-fermentation by *Pichia kudriavzevii* TY1322 only.

The *Pichia kudriavzevii* strain was developed in our laboratory by mutagenesis from a strain originally isolated from a traditionally fermented food product, based on cereals, produced and collected in Tanzania [150, 235]. Our results highlight the importance of microbiological knowledge and biotechnological approaches when using fermentation as a method to reduce the content of phytate, since fermentation per se will not always decrease phytate. Choosing appropriate strains capable of both production and extracellular release of phytase, strain combinations, optimal conditions during fermentation and strain improvement by biotechnological methods are vital to achieve a product with high mineral bioavailability through fermentation. Upregulation of phytase expression is another approach to improve strains aimed for phytate degradation. Due to the large potential of fermentation as a method to develop plant-products with high mineral bioavailability, utilization and optimization of fermentation should be encouraged in order to develop new plant foods with high and bioavailable nutritional properties.

### 5.2.3 Probiotic potential

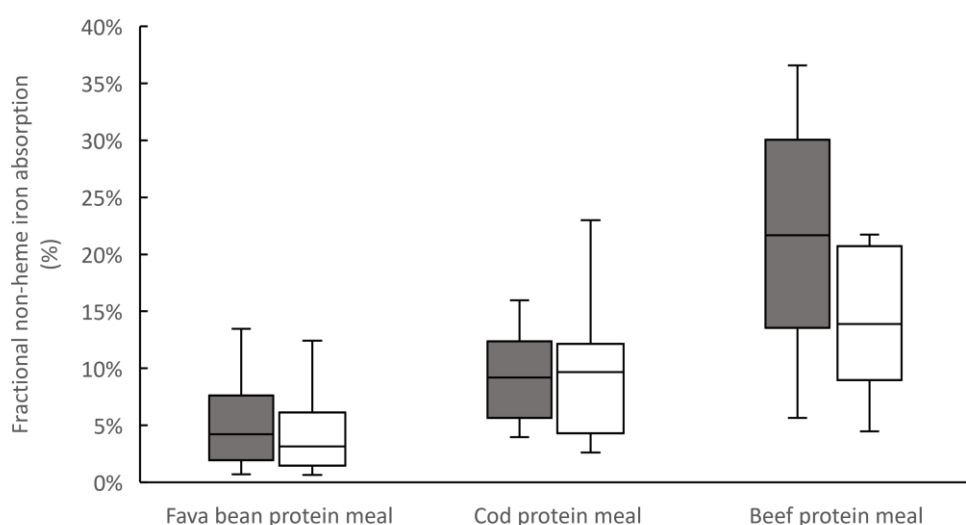
Tolerance to low pH among the 25 unique strains isolated in Study I varied. Only strains belonging to *B. coagulans*, and four strains belonging to *P. pentosaceus*, survived incubation. The *B. coagulans* strains showed an exponential growth, while *P. pentosaceus* remained on the same level of CFU throughout incubation. Across genera, phenol tolerance was in general high while the bile tolerance was inter-specific. Strains resilient to low pH, bile and phenol is thought to survive a passage through the gastrointestinal tract and hence potentially exert beneficial effects to the host. Five of the isolated strains met this prerequisite, three belonging to *B. coagulans* and two belonging to *P. pentosaceus*.



## 5.3 Results from clinical trials

### 5.3.1 Iron absorption from extracted fava bean, beef and cod protein

We found significant differences in the amount of non-heme iron absorbed from beef, cod and texturized fava bean protein meals (**Figure 6**).



**Figure 6. Box plot of adjusted and unadjusted non-heme iron absorption, data combined from two clinical single meal trials: Trial 1: texturized fava bean vs beef protein; Trial 2: texturized fava bean meal vs cod protein**

Presented absorption of texturized fava protein is based on combined data from Study 1 and Study 2. Grey boxed represent data adjusted to a 40% reference dose absorption, white boxes represent unadjusted data. Adjusted and unadjusted data based on combined data from absorption quota and WBC was significantly ( $p < 0.05$ ) different between each of the three meals, analyzed by Mann-Whitney U test.

Non-heme iron absorption, measured as  $^{59}\text{Fe}/^{55}\text{Fe}$  ratio in erythrocytes, from meals containing beef or cod protein was 4.2 and 2.7 times higher, respectively, compared to a meal with texturized fava bean protein. Combined data from WBC and erythrocyte analysis showed that the absorption of non-heme iron was 9.2% from the cod protein meal (9.7% unadjusted), 21.7% from beef protein meal (13.9% unadjusted) and 4.2% from texturized fava bean meal (combined data from Trial 1 and Trial 2. Unadjusted 3.2%). Apart from the non-heme iron, a large contribution to total iron absorbed can be

expected from heme iron in the beef and cod proteins. Approximately 40 % of the total iron in these animal proteins sources was found to be heme iron, from which up to 25% is considered to be absorbed [236]. Based on this assumption the total amount of iron absorbed was estimated to 0.62 mg from the beef protein meal (out of 2.7 mg total iron), i.e. 23%, 0.36 mg from the cod protein meal (out of 2.3 mg total iron), i.e. 16% and 0.16 mg from the fava bean meal (out of 3.7 mg total iron) i.e. 4%. Compared to the daily iron need of women of fertile ages, which is estimated to 2.22 mg/day (90<sup>th</sup> percentile) according to the Nordic Nutrition Recommendations [237], the contribution from the fava bean meal was 7.0%. The contribution to the daily need of iron from the beef protein meal was 28.0% and 16.3% from the cod protein meal. Despite having the highest amount of total iron, the actual iron absorbed from fava bean meal was significantly lower compared to the beef and cod protein meals. Our results show difficulties covering daily iron need with plant protein extract products as a substitute to beef or cod meals, especially for women of fertile ages as well as growing individuals with high iron needs. This is an important finding as the majority of meat substitutes currently on the market are based on legume protein extracts, with extrusion as the principal technique for building structure [112]. This further confirms the relevance of addressing the bioavailability of nutrients in plant products, rather than only the content.

#### *5.4. Scenario calculations*

Two scenario calculations were made in order to estimate the effect of a 100%, and a 50% exchange of current meat intake into selected alternatives analyzed in the work of this thesis.

According to the Swedish national dietary survey Riksmaten, Swedish women of fertile ages have a total meat intake of 88g per day on average [238]. Total meat includes red meat, poultry, offal, blood products and sausages. According to the Nordic Nutrition Recommendations, the daily need of iron is 2.22 mg per day for women of fertile ages [237]. The contribution to the daily iron need from current meat intake was calculated to 18% (Table 7). Calculations were based on a non-heme iron absorption of 20% from meat, similar to what has previously been reported by Hallberg et al [239] and Layrisse et al [83], and an assumption of 25% absorption from heme iron, as previously reported by Monsen et al [240].

Calculations show that a total exchange of meat would result in a 41-82% decrease of iron absorbed, compared with what was expected from the meat fraction, depending on the product

used for substitution (**Table 7**). Tempeh was calculated to have the least negative effect on iron absorbed, and a 50% reduction of meat substituted with tempeh would result in a 20% decrease of iron absorbed (**Table 8**). Products based on protein extracts were calculated to result in the most markedly decrease of absorbed iron compared with current meat intake. A total exchange from current meat intake among Swedish women into the extracted protein product Pea Schnitzel analyzed in Study III was calculated to result in a decrease of 73% of absorbed iron from what was expected from the meat fraction, as the extracted protein product was calculated to contribute with 4.6% of the daily iron need (Table 7).

Apart from containing a minimum amount of the specific nutrient, the condition “the nutrient for which the claim is made is in a form that is available to be used by the body” has to be fulfilled for a permitted nutritional claim, as stated by the EU regulations on nutrition claims [241]. Hence, a nutrition claim on iron used for a product with a high phy:Fe molar ratio, such as the products analyzed in Study III, can be argued as not permitted. Such a claim can also be seen as misleading and negative for a consumer aiming at substituting meat, which has a high bioavailability of iron, since it is not possible for the consumer to evaluate the nutritional contribution of such a product.

**Table 7.** Calculation of Scenario 1, a total exchange of current Swedish meat intake into four alternatives: 1-3 are products analyzed in Study III, and alternative 4 is replacement of meat with the fava bean test meal of Study IV

Reference diet		Scenario 1, 100% exchange of meat into:			
Swedish national diet		Alternative 1	Alternative 2	Alternative 3	Alternative 4
88g of:	Total meat	Protein extract product	Mycoprotein	Tempeh	Fava bean test meal
Protein (g)	19.7	7.1	13.6	5.9	16.6
Zinc (mg)	2.8	1.5	5.9	1.2	1.6
Total iron (mg)	1.7	2.0	0.4	1.8	1.8
Non-heme iron (mg)	1.0	2.0	0.4	1.8	1.8
Heme iron <sup>1</sup> (mg)	0.7	0	0	0	0
Phytate (mg)	0	1013	0	21	805
Calculated inositol Phosphates <sup>2</sup> (mg P)	0	286	0	6	227
Dietary fiber (g)	0	5.5	5.3	6.3	5.2
Salt (mg)	0.3	2.1	0.7	0.9	
Saturated fat (g)	2.3	1.1	0.2	0.9	0
Assumed non-heme iron absorption <sup>3</sup>	20%	5%	15%	12.5%	4.2%
Assumed heme iron absorption <sup>4</sup>	25%	-	-	-	-
Calculated iron absorbed (mg)	0.37	0.10	0.07	0.22	0.07
Difference in iron absorbed <sup>5</sup>		-73%	-82%	-41%	-80%
Percentage of daily iron need <sup>6</sup> (2.22 mg)	16.7%	4.6%	3.0%	9.9%	3.3%

Contribution of nutrients from the daily meat intake, and from the meat alternatives respectively, was calculated and shown in the table above

Total meat include meat from beef, pork, lamb, game, horse, poultry, offal, blood products and sausages. Red meat, offal and blood products contributes to the diet of Swedish women of fertile ages with 53g/dag, poultry with 20g/day and sausages 15g/day. Intake according to the population-based Swedish dietary survey Riksmaten (Swedish national diet) [238].

Nutritional composition of total meat intake was calculated based on the Swedish Food Agency food database [242] using the products "beef sirloin steak pan fried", "chicken meat cooked fried grilled" and "cooked falu sausage baked" as representative products for each meat category, as reported by Riksmaten.

<sup>1</sup>Heme iron was calculated based on the general assumption that 40% of the total iron in meat consist of heme iron [243]

<sup>2</sup>A phytate molecular weight of 660 g/mol was used

<sup>3</sup>Non-heme iron absorption from meat was assumed to be 20%, similar to previously reported [83, 239]. Absorption of non-heme iron from protein extract product "Pea Schnitzel" and the Tempeh product "Tempeh burger" was calculated based on the dose-response curve developed by Hallberg et al 1989 [136], and applied by Brune et al [149]. Non-heme iron absorption from texturized fava bean test meal was calculated based on the adjusted absorption value from the human trial of Study IV

<sup>4</sup>Heme iron absorption was assumed to be 25%, according to the previous work by Monsen et al [240]

<sup>5</sup>Compared with the contribution from meat in the reference diet

<sup>6</sup>For women of fertile ages, as according to the Nordic Nutrition Recommendations [237]

**Table 8.** Calculation of Scenario 2, a 50% exchange of current Swedish meat intake into four alternatives: 1-3 are products analyzed in Study III, and alternative 4 is replacement of meat with the fava bean test meal of Study IV

	Reference diet Swedish national diet	Scenario 2, 50% exchange of meat into:				
		Contribution from Total meat (44g)	Alternative 1 Protein extract product (44g)	Alternative 2 Mycoprotein (44g)	Alternative 3 Tempeh (44g)	Alternative 4 Fava bean test meal (44g)
Protein (g)	19.7	9.8	3.5	6.8	2.9	8.3
Zinc (mg)	2.8	1.4	0.7	2.9	0.6	0.8
Total iron (mg)	1.7	0.8	1.0	0.2	0.9	0.9
Non-heme iron (mg)	1.0	0.5	1.0	0.2	0.9	0.9
Heme iron <sup>1</sup> (mg)	0.7	0.3	0	0	0	0
Phytate (mg)	0	0	506	0	11	402
Calculated inositol Phosphates <sup>2</sup> (mg P)	0	0	143	0	3.0	113,0
Dietary fiber (g)	0	0	2.7	2.6	3.2	2.6
Salt (mg)	0.3	0.2	1.1	0.4	0.4	
Saturated fat (g)	2.3	1.2	0.6	0.1	0.4	0
Assumed non-heme iron absorption <sup>3</sup>	20%	20%	5%	15%	12.5%	4.2%
Assumed heme iron absorption <sup>4</sup>	25%	25%	-	-	-	-
Calculated iron absorbed (mg)	0.37	0.19	0.05	0.03	0.11	0.04
Calculated iron absorbed, sum (mg)		-	0.24	0.22	0.30	0.22
Difference in iron absorbed <sup>5</sup>		-	-36%	-41%	-20%	-40%
Percentage of daily iron need <sup>6</sup> (2.22 mg)	16.7%	8.3%	10.6%	9.8%	13.3%	10.0%

Table 8, Figure legend. Contribution of nutrients from the daily meat intake, and from the meat alternatives respectively, was calculated and shown in the table above

Total meat include meat from beef, pork, lamb, game, horse, poultry, offal, blood products and sausages. Red meat, offal and blood products contributes to the diet of Swedish women of fertile ages with 53g/day, poultry with 20g/day and sausages 15g/day. Intake according to the population-based Swedish dietary survey Riksmaten (Swedish national diet) [238].

Nutritional composition of total meat intake was calculated based on the Swedish Food Agency food database [242] using the products "beef sirloin steak pan fried", "chicken meat cooked fried grilled" and "cooked falu sausage baked" as representative products for each meat category, as reported by Riksmaten.

<sup>1</sup>Heme iron was calculated based on the general assumption that 40% of the total iron in meat consist of heme iron [243]

<sup>2</sup>A phytate molecular weight of 660 g/mol was used

<sup>3</sup>Non-heme iron absorption from meat was assumed to be 20%, similar to previously reported [83, 239]. Absorption of non-heme iron from Protein extract product "Pea Schnitzel" and the Tempeh product "Tempeh burger" was calculated based on the dose-response curve developed by Hallberg et al 1989 [136], and applied by Brune et al [149]. Non-heme iron absorption from texturized fava bean test meal was calculated based on the adjusted absorption value from the human trial of Study IV

<sup>4</sup>Heme iron absorption was assumed to be 25%, according to the previous work by Monsen et al [240]

<sup>5</sup>Compared with the contribution from meat in the reference diet

<sup>6</sup>For women of fertile ages, as according to the Nordic Nutrition Recommendations [237]

## 6. GENERAL DISCUSSION

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The overall aim of this thesis was to investigate the nutritional and antinutritional composition of plant-based food products aimed at replacing meat, study strategies for improved nutritional quality and compare a plant-based model meal with meat and fish with respect to iron absorption.

Results presented in this thesis clearly demonstrate shortcomings when it comes to iron and zinc bioavailability in meat substitutes and fava bean cultivars, as has long been raised as a concern regarding plant-based diets.

As previously discussed, the quality of a plant-based diet is of higher relevance compared with simply being plant-based. Many of the initial studies where the impact of plant-based diets on different health outcomes were investigated, recruited study populations with healthier lifestyle patterns compared with the general population, such as Seventh-day Adventists, Buddhist monks and traditional vegetarians and vegans [244-249]. Despite the large body of evidence on the association of low quality plant-based foods and negative health effects (such as refined carbohydrates, added sugars and starchy foods), only recently studies comparing a “low-” and a “high” quality plant-based diet have been published. Unsurprisingly, significant differences in health outcomes between “healthy” compared with “unhealthy” plant-based food patterns have been shown [34, 250-255].

Since we found large variations in the nutritional composition and quality of fava bean cultivars and meat substitutes, the nutritional gain from a diet composed of these products will substantially differ depending on the products included. Many of the products analyzed in this thesis contained a high amount of salt and saturated fat, while others would fit better into nutritional guidelines of these nutrients. As an example, a portion of an average mycoprotein product, which had significantly lower salt contents compared with other categories, would contribute with 1.0 gram of salt (16.7% of maximum daily intake according to the Nordic Nutrition Recommendations [256]), while a same size portion of the product “Pea schnitzel” that had the highest content of salt would contribute with 3.6g of salt. This corresponds to 59.3% of the recommended maximum daily intake of salt, based on a limit of 6 grams of salt per day [256], which can be considered a substantial contribution.

The quality and composition are not only defined by cultivar, crop and added compounds such as salt and which fat that has been added, but also on the processing methods

and fractions used. While added fat and salt easily can be improved by changes in the formulation of a product, process effects on nutritional quality require deeper knowledge.

For example, amino acid composition has previously been shown to be affected by extraction and extrusion of plant protein, which probably explained the results on amino acid composition in the group of meat substitutes consisting of both cereal and legume protein extracts in Study III. These products did not have complementary amino acid profiles, which otherwise could be expected from a combination of the two plant sources. Products based on extracted and/or extruded plant protein hence cannot be considered equal to the raw material from which they were produced from, with regard to nutrition and health. Moreover, we found very high contents of phytate in meat substitutes based on legume protein extracts, which can be explained by previous findings on the accumulation of phytate during protein extraction.

Phytate has been a large focus in this thesis, mainly due to its potent inhibitory effect on mineral absorption. The negative effect of phytate is dose dependent, with a notable effect even at very low levels. Therefore, reduction of phytate content has many times been emphasized as necessary for the improvement of mineral bioavailability in plant foods as stimulating factors, such as ascorbic acid and the meat factor, only can counteract a certain amount of phytate.

Fermentation is often emphasized as a method to reduce the content of phytate, which is true if optimal microorganisms and conditions are being used. In Study II, a lactic acid bacterial strain belonging to *Pediococcus pentosaceus* was not able to reduce the content of phytate in a fava bean drink, while the phytase producing yeast *Pichia kudriavzevii* degraded phytate in mono-fermentation and even more efficiently in co-fermentation with the *P. pentosaceus* strain. These results exemplify the complexity of fermentation, and the need for integration of nutrition and biotechnology in the area of product development, especially for the development of products aimed at substituting animal products that has a high bioavailability of nutrients.

Products and cultivars investigated in this thesis had very low estimated bioavailability of both iron and zinc, due to the previously mentioned high content of phytate. Mycoprotein products were an exception as the single group of meat substitutes which could be considered a good source of one of the minerals, namely zinc. The theoretical low bioavailability of iron was confirmed by the non-heme iron absorption clinical trial conducted in Study IV, where extracted and texturized fava bean protein was compared with beef and cod protein in single meals. Absorption of non-heme iron, measured as erythrocyte incorporation ratio, from a beef protein meal was 4 times higher compared to texturized fava bean meal, and



the absorption from cod protein meal was 3 times higher compared to the fava bean meal. Total iron absorbed, including heme iron, differed greatly between animal protein meals and texturized fava bean meal. A total substitution of meat among Swedish women, with meat substitutes analyzed in this thesis was calculated to result in a 41-82% decrease of iron absorbed, compared with what was expected from the meat fraction.

Overall, results show that the nutritional gain from consuming meat substitutes and fava bean is significantly affected by the product, cultivar and processing method used.

It is clear that there is a large need to include nutritional knowledge in the development of plant protein extraction methods aimed at providing ingredients for foods suitable in the protein shift, with a high nutritional quality as well as a low climate and environmental footprint.

Further on, our results call for updates in the interpretation of regulations on nutrition claims. As an example, there are recommendations on theoretical Fe and Zn bioavailability, presented as maximum phy:mineral molar ratios, suggested by EFSA, WHO and FAO. Implementing these recommended molar ratios as threshold for approved nutrition claims should be considered as this would 1) be in line with the European Regulation on nutrition and health claims, 2) create incentive to produce plant products with a substantially better bioavailability of Fe and Zn and 3) help reduce negative effects caused by low Fe and Zn status, especially among vulnerable groups. It is crucial to include nutrition and bioavailability considerations to the protein shift and in the development of new recommendations as well as policies. As an example, Nordic Nutrition Recommendations (NNR) calculate recommended daily intake of iron based on a bioavailability of 15%, which corresponds to a diet containing meat. There are no specific recommendations for low-bioavailability diets [237].

While climate has a vital role in the complex concept of sustainability, health and nutrition are other essential aspects of the same concept. There are 17 global sustainable development goals (SDGs), some of which inherently stand in conflict with each other. Integration of the 169 targets set for the SDGs require deep cross-sectional knowledge, broad collaborations, and a fundamental understanding of the interactions between different aspects of sustainability. Sustainability cannot be achieved using only parts of the puzzle, at the expense of the rest.

## 7. CONCLUSIONS

This thesis evaluated nutritional quality, estimated bioavailability and non-heme iron absorption in women of fertile ages from products developed for substitution of meat. The results reveal poor nutritional profile and low estimated iron bioavailability of a majority of meat replacement products, which may constitute a risk, particularly in the light of increased consumption among women of fertile age and adolescents. Nutrition and health should be made more visible as sustainability aspects in the discussion of the protein shift.

More precisely, results showed that:

- Fava bean varieties cultivated in Sweden has a very low estimated bioavailability of iron and zinc, except for cultivar Sunrise which simultaneously had a high content of iron and zinc, and low content of phytate. Cultivar Lynx was another low-phytate cultivar. Nutritional and antinutritional content varies largely between cultivars of fava bean, and the nutritional gain from fava beans is significantly affected by the cultivar. Low phytate cultivars can serve as potential starting material for further work on fava bean.
- Fermentation with a mixture of phytase secreting yeast and LAB can significantly reduce the content of phytate in a fava bean drink to improve mineral bioavailability. It could thus be an attractive approach to provide fava bean drinks with good nutritional and environmental properties. The magnitude of phytate degradation is affected by both strain choice and fermentation conditions and needs to be optimized.
- Development of nutritiously adequate meat substitutes is needed to avoid negative effects following substitution of meat. Improvement of product formulations can be used as an easy measure to reduce the content of salt and saturated fat. Method development, and implementation of existing processing methods, aiming at increasing nutrient bioavailability should be prioritized. The nutritional effect of plant protein extraction and extrusion has not received enough emphasis. Meat substitutes commonly found on the Swedish market have in general a high content of phytate and a very low estimated bioavailability of iron and zinc, with the exception of mycoprotein products (high estimated zinc bioavailability) and tempeh. Producers of meat substitutes commonly found on the Swedish market use nutritional claims on iron that appear not in line with European Regulations on nutrition and health claims since the nutrient for which the claim is made has to be in a form that is available to be used by the body, although this has so far not been legally tested.

- A texturized fava bean protein meal has a markedly lower iron bioavailability in healthy females compared to a meal with beef or cod protein. Non-heme iron from cod protein meal was absorbed 3.0 times higher compared to texturized fava bean meal, and from beef protein meal the absorption was 4.7 times higher. A dietary shift from beef to fava bean protein may therefore increase the risk of developing iron deficiency.
- A total exchange of the current meat intake among Swedish women, substituted with plant-based products analyzed in this thesis, was calculated to result in a 41-82% decrease of iron absorbed, compared with what was expected from the meat fraction. Products based on protein extracts were calculated to result in the largest decrease of absorbed iron compared with current meat intake, and tempeh was calculated to result in the smallest decrease.

## 8. FUTURE PERSPECTIVES

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Results presented in this thesis clearly demonstrate poor nutrient profile and mineral bioavailability in fava bean and meat substitutes, as has long been raised as a concern regarding a plant-based diets. Several questions have arisen during the work of this thesis. Apart from implementation and integration of nutritional aspects of sustainability, future work should include:

- A deeper investigation of the typical Western plant-based diet. Since the main focus in the majority of cohorts investigating plant-based diets has been on high quality food patterns followed by a minority, often health-conscious individuals, composition of modern plant-based food patterns needs to be investigated in order to give answer to where the protein shift has currently led Western populations. Additional product categories aiming at substituting animal products, for example non-dairy, should be critically evaluated in terms of composition, quality and health effects.
- Randomized control intervention trials on whole diets. Expanding single meal absorption studies to whole diet studies, as well as long term studies on the effect of plant-based food patterns on micronutrient status are needed.
- Observational studies on individuals growing up on plant-based diets. Since food patterns are changing from having high-bioavailable to low-bioavailable nutrients following the protein shift, nutritional and health effects should be investigated in children and adolescents following Western plant-based food patterns. Additionally, investigation is lacking on the effects on nutritional status, health outcomes and development of children where their mothers have been following plant-based food patterns during pregnancy.
- Development of bioprocessing techniques used for improved nutritional quality of substituting products. Work should include investigation of strain combinations, substrate optimization and optimization of fermentation conditions and development of phytase expressing strains; upregulation of phytase activity and ensuring extracellular release of phytase.
- Development of modelling systems for evaluating sustainability within the food sector. While the main focus of sustainability currently revolves around climate and land use, nutrition and health are important aspects of sustainability. Integration of nutrition and health, taking into account bioavailability and local conditions, into modelling systems aiming at evaluating sustainability of a food product, food

production system or dietary patterns, is essential in order to avoid negative consequences of the protein shift.

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