School of Public Health

Nutritional Composition of (Australian Sweet Lupin) Natto and Tempeh: Effect of Source and Fermentation Time

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Author's Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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Abstract

Australian sweet lupin (ASL) (*Lupinus angustifolius*) is a potentially important source of protein and dietary fibre with a unique nutritional composition different to that of most other legumes but somewhat similar to soybean but with much lower lipid levels. Traditionally used as animal feed, lupin has captured the attention of researchers worldwide due to its health promoting effects and potential use as a functional food.

Lupin seed is rich in minerals, but their bioavailability is low due to the presence of mineral chelating dietary fibres, and antinutritional factors such as phytates and polyphenols. Effective removal of these anti-nutritional compounds thus improving bioavailability of minerals might be obtained by biological food processing, such as fermentation. Natto, is an indigenous traditional Japanese food which is produced from whole seed soybean fermented with the bacteria *Bacillus subtilis,* while tempeh is an indigenous Indonesian soybean fermented with the mould *Rhizopus oligosporus.* Given the somewhat similar composition of lupin seeds and soybean, the lupin has great potential for the manufacture of natto and tempeh analogues. Research presented in this thesis demonstrates that lupin based natto and tempeh with high quality can be produced. Further, this thesis aimed at understanding the importance of fermentation time and source of lupin [whole seed (WS) or dehulled seed (DHS)] on the physical, chemical and organoleptic properties of the final natto and tempeh, specifically the proximate and dietary fibre composition, protein digestibility and mineral bioavailability and content of the phytoestrogenic

isoflavones daidzein and genistein. The major findings of this thesis are that levels of protein, fat, and soluble dietary fibre are increased with fermentation and that dehulled lupin natto and tempeh were nutritionally better than those made from whole seed lupin. Daidzein and genistein which is low in raw lupin compared to soybean, increased on fermentation and there was a significant effect in fermentation time x lupin source interaction effect.

There was a significant effect of fermentation time on mineral bioavailability of lupin natto and tempeh from lupin dehulled seed. Fe bioavailability of dehulled seed natto was significantly higher 24 h following fermentation. Zn bioavailability was significantly higher at 36 h in whole seed lupin natto but at 24 h in dehulled seed natto. Future studies could incorporate these lupins fermented products into diets in clinical trials to assess their value to human nutrition and protection from chronic disease.

Findings of this thesis show that, lupin could be used as substitute for soybean in the Asian traditional fermented legume food industry. Due to cost effectiveness of using lupin seed as a raw material for natto and tempeh, lupin could be exported to these countries for their manufacture. This could benefit lupin famers and the food industry. Furthermore, given the lupin natto and tempeh contained significantly higher protein content, and bioavailability, higher soluble dietary fibre and increased bioavailability of some minerals compared to the unfermented seed, lupin natto and tempeh may be promoted as health functional food to assist consumer nutrition.

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ABBREVIATIONS

ACE	Angiotensin converting enzyme
AD	Alzheimer’s Disease
ANOVA	Analysis of variance
AOAC	Association of Analytical Chemists
ASL	Australian sweet lupin
ATCC	American type culture collection
CVD	Cardiovascular disease
DHS	Dehulled seed
DHLT	Dehulled lupin tempeh
DHLN	Dehulled lupin natto
db	Dry basis
FAO	Food and Agriculture Organization
GABA	Gamma amino butyric acid
IDF	Insoluble dietary fibre
IFT	Institute of Food Technologists
ISC	Indonesian starter culture
h	Hour
HDL-C	High density lipoprotein cholesterol
HPLC	High performance liquid chromatography

IVPD	In vitro protein digestibility
KDA	Kilodalton
LDL-C	Low density lipoprotein cholesterol
LKF	Lupin kernel fibre
LPI	Lupin protein isolate
NSP	Non-starch polysaccharides
PUFA	Polyunsaturated fatty acid
RBA	Rose Bengal agar
RFO	Raffinose family oligosaccharides
RH	Relative humidity
SCFA	Short chain fatty acid
SD	Standard deviation
SDF	Soluble dietary fibre
SFA	Saturated fatty acid
TC	Total cholesterol
TDF	Total dietary fibre
TA	Texture analysis
USC	USA starter
UK	United Kingdom
USA	United States of America
USDA	United States Department of Agriculture
VAS	Visual Analogue Scale

WA	Western Australia
WS	Whole seed
WSLT	Whole seed lupin tempeh
WSLN	Whole seed lupin natto

CHAPTER 1
GENERAL INTRODUCTION

1.1 Background

Growing interest in some foods that might improve health has resulted in the use of the term “functional foods”. As defined by Institute of Food Technologists (IFT), experts set forth that functional foods describe “*foods and food components that provide essential nutrients often beyond quantities necessary for normal maintenance, growth, and development, and/or other biologically active components that impart health benefits or desirable physiological effects”* (IFT 2017). Nutrition and health are intimately linked, and medicinal properties of foods have been known for generations. Hippocrates’s famous saying, “*Let food be thy medicine and medicine be thy the food*” is frequently quoted to explain the ability of foods and food components to reduce disease risk (Milner, 2002). While medicinal foods have been used to treat diseases for a long time, recent research findings and scientific knowledge on bioactive compounds of many foods are now being used to formulate novel functional foods (Singh, 2016). At present, nutritional science has taken great steps in promoting consumption of functional foods for optimal health and reducing the risk of disease. However, the development, marketing and regulation of these new foods should be monitored and controlled by sound science to ensure their safety and efficacy. Moreover, advances in food technology, greater consumer demand and new evidence-based science linking diet to disease, has presented an unrivalled opportunity to address public health issues through diet. The opportunity for developing functional foods will be further broadened, when additional bioactive compounds are identified. Early nutrition research mainly focused on various vitamin and

minerals for preventing deficiency diseases. However similar research is needed to identify the role of bioactive compounds, as these areas still need more attention. Research has demonstrated that specific foods and isolated food components can reduce the risk of certain disease; from the effect of soluble fibre from oats and legume protein from soy on reducing coronary heart disease risk, to the protective effect of high calcium foods on osteoporosis. According to the traditional paradigm, the mere purpose of food was promoting normal growth and development. Since current medical treatments may not have their optimal desired effect, it is worthwhile to study functional foods in detail which may produce better health out come when combined with drugs (Johnson, Clements, Villarino, & Coorey, 2017).

Lupin, a high protein and high fibre legume seed, is considered a functional food ingredient since published research indicates that its consumption has numerous health benefits including anti-diabetic, anti-dyslipidaemic, anti-hypertensive and other cardiovascular disease protective effects (Sirtori et al., 2004; Marchesi et al., 2008; Lovati et al., 2012; Sirtori, & Arnoldi, 2009; Mousa, 2015). Different species of lupins have been used around the world for human food for over 6000 years (Schindler et al., 2011). Currently 80% of world lupin production is from Western Australia but still only 4% of world production is being used as a human food (Belski et al., 2011). Australian sweet lupin (ASL, *Lupinus angustifolius)* is the predominantly cultivated species in Australia. As recently reported (Johnson et al., In press) Lupin seed contains 32.2-42.0 g/ 100 g^{-1} dry basis db. protein depending on the species, which is similar to soybean but higher than other legumes such as chickpea (28.0 g 100 g^{-1} db.), lentil (28.6 g 100 g^{-1} db.) and dry pea (23.3 g 100 g^{-1} db) (Roy, Boye, & Simpson, 2010). Similar to most other legumes, lupin does not have a nutritionally complete or balanced amino acid

profile with scarcity of the sulphur containing amino acids methionine and cysteine (da Silva, Celeghini, & Chang, 2011). The oil content of lupin varies 5.5-13.0 (g 100 g^{-1} db.) depending on the species (Fleetwood & Hudson, 1982). Lupin kernel flour of *L. angustifolius* has 19% saturated fat, 33% is monounsaturated and 48% polyunsaturated (Kouris-Blazos, 2016). The main saturated fatty acid present is palmitic acid whereas the unsaturated fatty acids are oleic and linoleic (Rumiyati, 2010). The comparable protein content and low cost of lupin seed compared to soybean has led to increased interest in its potential as a human food (Johnson et al., 2017) in particular for manufacture of analogues of fermented soy foods. However, a fuller nutritional benefit from lupin may be obtained when fermented, as fermentation of other legumes such as soybean has shown improvement in their nutritional value, eliminates anti-nutritional factors, and increases digestibility and bioavailability of nutrients. Thus, fermentation may enhance the potential functional food properties of lupin seed by increasing its nutritional quality.

Campbell-Platt (1987) defined fermented food as “those food that have been subjected to the action of microorganism or enzyme so that desirable biochemical changes cause significant modification in the food. Also, fermentation can be defined as modifying food by a microorganism that can grow and reproduce and make use of part of the food as a substrate and enrich the food with the products of their metabolism (Fernandez-Orozco et al., 2007). The process of fermentation, through use of bacteria, mould, or yeast essentially converts a complex food into some simpler products, improving many of its nutritional qualities (Deshpande, 2000). Fermentation is an important method of food preservation as it eliminates spoilage microbes and has been widely used to modify food to enhance nutrient composition and bioavailability. For instance,

fermented soybean foods such as tempeh and natto, have more simpler forms of macronutrients than the raw soybean they are made from (Murooka & Yamshita, 2008). Decreased cooking times, detoxification and flavour, aroma and texture enhancement are other important roles of fermentation of food (Deshpande, 2000). Fermentation of lupin (Frias, Miranda, Doblado, & Vidal-Valverde, 2005) and other legume seeds, flour and its fractions has been previously reported using different microorganisms such as lactic acid fermentation by the bacteria *Lactobacillu*s or *Streptococcus* (Kanekar, Joshi, Sarnaik, & Kelkar, 1992; Starzyńska Janiszewska, Stodolak, & Mickowska, 2014), *Saccharomyces spp. and Fermivin spp.*, or mould of *Aspergillus spp.* (Hong, Lee, & Kim, 2004; Sardjono, Zhu, & Knol, 1998). In contrast, alkaline fermentation forming a ferment medium with a pH>7 can occur during fermentation by some microorganisms. In this case, protein in the material is utilized as energy by the bacteria/mould and ammonia is produced as a by-product.

One widely consumed example of alkaline fermentation is that of soybean and *Rhizopus oligosporus* which forms the traditional Indonesian fermented food tempeh. Nout and Rombouts (1990) defined that tempeh of good quality is a firm and sliceable mass of cooked particles enfolded, infiltrated and compacted by a non-sporulated mycelium of *Rhizopus spp.* with an attractive texture and flavour. Generally, soybean kernels that have been soaked and cooked to soften them are inoculated with the *R. oligosporus* mould. In order to obtain good quality tempeh, fermentation parameters such as sufficient oxygen, moisture (85%) and temperature (37 °C) must be controlled and good quality product can be produced within 24 h (Steinkraus, Hwa, Van Buren, Provvidenti, & Hand, 1960; Deshpande, 2000). Using different processing methods and conditions varying compositions and acceptability of final product of tempeh from many legume and grain (barley,

black gram, chickpea, cow pea, snow peas, lupin, oats and wheat) have been reported. Therefore, there is a need of studying, sources and processing methods, product quality characteristics namely moisture, pH, firmness, ammonia content and total plate count and acceptability of tempeh from lupin seeds. The production of a tempeh analogue from lupin seed has been reported earlier (Agosin, Diaz, Aravena, & YaÑEz, 1989; Fudiyansyah, Petterson, Bell, & Fairbrother, 1995a; R. Coorey, Ho, & Jayasena, 2011). Fermentation of soybean, has been reported to increase protein content, eliminate the antinutritional factor trypsin inhibitor and reduce peptide size (Hong, Lee, & Sung, 2004). Fermentation of soybean and lupin by Aspergillus has been shown that fermentation profile and nutritional value of lupin has similarities to soybean and therefore potential replacement of soybean in fermented food by lupin should be achievable (Sardjono et al., 1998). Antioxidant activity of black soybean fermented with *Bacillus spp. Aspergillus spp.* and *yeast* was much higher compared to unfermented beans. *Bacillus* was the most effective compared to Aspergillus fermentation (Qin, Jin, & Park Heui, 2010). Fermentation with *R. oligosporus* increased antioxidant activity of fava-beans, soybean and oats (Berghofer, Grzeskowiak, Mundigler, Sentall, & Walcak, 1998). Substitution of soybean with lupin seeds at different percentages in tempeh has been reported with organoleptic evaluation showing there was no significant difference between the control (soybean) and lupin-soy based product (Fudiyansyah et al., 1995a). Fermentation has been reported as a way of increasing protein digestibility of many legumes (El Hag, El Tinay, & Yousif, 2002; Yousif & El Tinay, 2000) though generally raw lupin has higher protein digestibility compared to other similar legume seeds (Schoeneberger, Gross, Cremer, & Elmadfa, 1983). The *R. oligosporus* mould in tempeh has some beneficial effects on detoxification of lupin

seed as it degrades their potentially toxic quinolizidine alkaloids (Ortega-david & Rodríguez-stouvenel, 2013).

Fermentation improves the nutritional quality of lupin, as in the case of soybean there is a significant breakdown of phytic acid that otherwise can reduce mineral bioavailability and there is also a reduction in flatulence-causing oligosaccharides (Camacho et al., 1991). As reported by Kouris-Blazos(2016) lupin has high amount of dietary fibre (~30 g 100 g^{-1} db.) and hence it is distinctive among other legumes including soybean. Dietary fibre consists of a range of non-starch polysaccharides and the associated lignin and indigestible oligosaccharides. Also, as defined by Dingara (2012), dietary fibres are food components that cannot be digested in the human digestive system, hence reach the colon where they are fermented or provide bulk that can help maintain health. Due to relatively higher level of non-starch polysaccharides and oligosaccharides, (non-digestible highly fermentable carbohydrates) in lupin compared to other legumes, flatulence is noted as a problem associated with its consumption, however this can be overcome by fermentation (Refstie, Sahlström, Bråthen, Baeverfjord, & Krogedal, 2005) where these oligosaccharides are broken down (Refstie et al., 2005; Johnson, Chua, Hall, & Baxter, 2006).

In contrast to tempeh production, an alkaline fermentation of soybean by the bacteria *B. subtilis* forms the Japanese fermented traditional food natto (Sumi, Hamada, Tsushima, Mihara, & Muraki, 1987a). Natto originated in Northern Japan about 1000 years ago and similar products are found by different names in Asia namely shi (China), thua-nao (Thailand), chung-kook-jong (Korea) and tao-si (Philippines) (Wei, Wolf-Hall, & Chang, 2001). Good quality natto should be covered with white mucus, have characteristic flavour, soft in texture and sticky mass when mixing with a pair of chopsticks. The above characteristics of natto

are affected by various factors such as soaking, seed processing methods, fermentation time and strain of bacteria. As reported by Matsumoto and others (1993) the best quality natto was produced at 35 °C with an inoculation of 10^{4}-10^{6} cells g^{-1} or at 40 °C with 10^{2}-10^{4} cells g^{-1} of the stater culture. Similarly, natto prepared from lupin may have different characteristics when prepared under different conditions. However, the development of a natto analogue from lupin has never been investigated or documented before. Therefore, studies on *B. subtilis* fermentation of lupin seeds, to produce a novel natto analogue, require investigation.

Soybean and its fermented foods are rich in the phytoestrogens genistein and daidzein which exist as 7 O-glycosides (Sirtori, Arnoldi, & Johnson, 2005). Phytoestrogens are plant derived isoflavone compounds which have similarity in structure and function to human estrogen (Dixon, 2004). There is ongoing evidence for beneficial effects of phytoestrogens on glucose metabolism, lipid metabolism, osteoporosis and health issues related to menopause (Sirtori, Agradi, Conti, Mantero, & Gatti, 1977; Fujimoto et al., 1991; Anderson, Johnstone & Cook-Newell 1995; de Kleijn, van der Schouw, Wilson, Grobbee, & Jacques, 2002; Saha, Sadhukhan & Sil, 2014; Yang et al., 2010). However, these health benefits of soya and phytoestrogens is a controversial issue as number of research findings have indicated inverse results as well (Maxwell Parkin, 1989; Anderson et al., 1995; Yuan, Wang, Ross, Henderson, & Yu, 1995; Nestel et al., 1997; Greaves, Parks, Williams, & Wagner, 1999). Due to these negative findings health authorities of some countries have indicated maximum daily intake level for phytoestrogen (Sirtori et al., 2005). Fermentation (Haron, Ismail, Azlan, Shahar, & Peng, 2009; Bavia et al., 2012) and germination (Suparmo & Markakis, 1987; Dueñas, Hernández, Estrella, & Fernández, 2009) are processing

techniques in which the hydrolysis of isoflavone glycosides can occur as a result of digestion activities forming aglycones (Wu & Chou, 2009; Ferreira et al., 2011). Fermentation converts glycosylated isoflavone (genistin and daidzin) into their aglycone counterpart (genistein and daidzein) which eventually increase their bioavailability when ingested by humans (Izumi et al., 2000; Haron et al., 2009). However, the levels of bioactive compounds such as daidzein and genistein during fermentation of lupin tempeh and natto have never been investigated before. Also, no in depth analytical study, characterizing the full nutritional profile of lupin natto and tempeh has been conducted.

1.2 Aims and objectives

Overall objectives

The overall aim of this study was to develop novel lupin natto and lupin tempeh analogues from whole seed lupin and dehulled lupin kernels and characterize their physical and chemical properties. Also, it was an aim to understand the changes in nutritional and bioactive components that occur due to fermentation of Australian sweet lupin (ASL). In order to achieve this aim, fermentation by a bacteria *B. subtilis* to form a natto analogue and a mould *R. oligosporus*, to form a tempeh analogue were performed and the products were analysed for their nutritional and bioactive compounds and thus their potential as a functional food.

Specific objectives

1. To determine the effect of seed source (whole seed and de-hulled seed), seed processing methods (boiling and pressure cooking), strain of microorganisms (*B. sublitis*, and *R. oligosporus*) inoculum level and fermentation time on organoleptic properties, moisture, pH, hardness, bacteria population and ammonia content of lupin natto and tempeh.
2. To determine the effect of the different seed source (whole seed and de-hulled seed), and fermentation time (0-72 h) using standardised method (from aim 1) on nutritional composition and dietary fibre profile of raw lupin, lupin natto and lupin tempeh.
3. To study the effect of different seed source (whole seed and de-hulled seed), and fermentation time (0-72 h) using standardised method (from aim 1) on genistein and daidzein content.
4. To assess the effect of seed source and fermentation time on mineral composition, distribution and *in vitro* bioavailability of lupin natto and tempeh.

CHAPTER 2
REVIEW OF LITERATURE

2.0 Connecting Statement

This chapter serves as an introduction to the thesis, showing the background for the research that was done and reasons why this project was significant.

2.1 Introduction

Affordable sources of foods with high nutritional value are presently being sought in an effort to battle common chronic diseases such as diabetes mellitus, hypertension and cardiovascular diseases (CVD) (Hall, Thomas, & Johnson, 2005; Bertoglio et al., 2011; Dove et al., 2011). Ubiquitous interest in the possibility that selected foods might promote health has resulted in the inventing of the generic term functional food. Functional food can be defined as one that encompasses potentially healthful products, including “any modified food or food ingredient that may provide a health benefit beyond that of the traditional nutrients it contains” (Institute of Food science and Technology, 2017). Lupin is valued for its excellent nutritional composition i.e low sugar content, high protein, complex carbohydrates or dietary fibre, vitamins, antioxidants, nutritionally essential minerals for human nutrition (Johnson, 2017) and most importantly it can be considered as a food ingredient with functional food effect (Capraro et al., 2011; Hassan, Rasmy, El-Gharably, El-Megied, & Gadalla, 2014; Rosa Lovati et al., 2012). Lupin seeds and flour have been used in a limited amount as healthy nutritious food for many centuries (Osman, Osman, Mahmoud, Romeilah, & Fayed, 2011) and recently, it has been used in a wide range of food such as tofu

and noodles (Jayasena et al. 2011; Jayasena, Leung, & Nasar-Abbas, 2010), ice-cream (Yap, 2006) and in fermented milk (Martinez-Villaluenga and Gomez, 2007). More recently scientific data indicates that lupin based foods have a wide range of benefits, such as anti-diabetic effect, hypolipidemic effect, fibrinolytic activity, anti-carcinogenic effect and are beneficial for bowel health (Sumi, Hamada, Nakanishi, & Hiratani, 1990; Magni et al., 2004; Johnson et al., 2006; Sirtori, Galli, Anderson, Sirtori, & Arnoldi, 2009; Fontanari, Batistuti, Cruz, Saldiva, and Arêas 2012; Hassan, Rasmy, El-Gharably, El-Megied, & Gadalla, 2014). However, unlike soybean, lupin seed contains only very low level of phytoestrogens (Khan, Karnpanit, Nasar-Abbas, Huma, & Jayasena, 2015). Therefore, lupin is not only a food ingredient with high nutritional value but also a potential functional food due to its potential disease protective ability. Lupin's comparable protein content and low cost compared to soybean has led recently to its increased consumption. However greater nutritional and health benefits might be achieved when lupin is fermented, as fermentation of soybean has been reported to eliminate anti-nutritional factors, increase digestibility and increase bioavailability of nutrients and may change phytochemical levels (Fernandez-Orozco et al., 2007; Fernandez-Orozco et al., 2008; Chang et al., 2009). Key examples of such fermented products are (a) natto which refers to the soybean based Japanese traditional food which is fermented with *subtilis* and (b) the soybean based Indonesian traditional food which is fermented with *Rhizopus oligosporus.* Currently there is a limited use of lupin in its fermented form although lupin tempeh is produced commercially. However, production of natto analogue from lupin has been rarely reported.

This review chapter covers the nutritional composition and evidence of the functional food value of lupin and the effect of fermentation on above qualities. The terms lupin, fermentation, natto, tempeh, protein digestibility and mineral bioavailability were used to search Medline®, Science Direct®, Springer Link® Web of Science® and Wiley online library®. One foundation article from 1960 was found and all other articles were from 1980 to the present date. Information, history of lupin origin, nutritional composition and health benefits are represented first. This will be followed by general discussion on fermentation for food use followed by an extensive overview of natto and tempeh type solid state fermentation methods. Finally, the nutritional and health benefits of natto, and tempeh will be discussed.

2.2 Lupin

Lupins (*Lupinus* L.) are members of the legume family (subfamily Papilionoidea) containing both herbaceous annual and shrubby perennial types with attractive long racemes of flowers. Its rich diversity of species can be grouped into Mediterranean and East African 'Old World' species and American 'New World' species (Kurlovich, 2002). In their natural state, lupins have adapted to the sub-arctic climates of Alaska and Iceland, the arid climates of east Africa and Mexico, and the sub-tropical parts of South America and the USA (Taranukho, 1980).

Being a legume, lupins fix atmospheric nitrogen via a rhizobium-root nodule symbiosis, which together with a deep root system, explains their tolerance of infertile soils. Lupins are often a pioneering species of disturbed ground as was illustrated by the rapid colonisation by *L. lepidus* on the barren landscape created by the volcanic eruption of Mt St Helens (Washington State, USA) in 1980 (Kurlovich,1989).

The common name for native *Lupinus* in North America is ‘lupine’, however ‘lupin’ is commonly used in Europe and Australia for both native and agricultural forms (Gladstones, 1974).

The potential for confusion is further illustrated with *L. angustifolius*. In the wild state it has blue flowers and is referred to as the ‘blue lupin’ in Europe. All agricultural cultivars of *L. angustifolius* in Australia have been bred to have white flowers, to distinguish them from their bitter undomesticated relatives. In the 1980s Australian farmers colloquially referred to them as ‘white lupins’ (which is the common name for *L. albus* in Europe). Scientists in Australia have encouraged the name ‘narrow-leafed lupin’ whilst industry often use ‘Australian Sweet Lupin’ for *L. angustifolius* (Gladstones,1974).

2.2.1 Ancient History and 20th Century Domestication

Lupins have a history in agriculture and as a food that traces back more than 2000 y. They were eaten by the early Egyptian and pre-Incan civilisations and promoted by Roman agriculturalists for their role in soil fertility (Taranukho, 1980).

Lupins were moved from their Mediterranean origins to northern Europe by Frederick the Great of Prussia in 1781 to improve the poor soils of northern Germany. By the 1860s the ‘garden yellow lupin’ was widespread across the acid sandy soils of the Baltic coastal plain for forage and green manuring (Gladstones,1974). The early 20th century saw the first steps taken to turn the lupin from a wild or semi-domesticated form into a modern crop plant. This work was pioneered by German scientists who screened thousands of lupin plants. Their goal was to cultivate a ‘sweet’ variety. The bitterness (due to a mixture of alkaloids) is undesirable in animal feed and human food and had prevented lupin’s widespread use for these purposes. The successful development of lupin varieties with the necessary ‘sweet gene’ paved the way for greater adoption of lupins in Europe and subsequently in Australia, where more sweet lupins are produced than anywhere else in the world (Gladstones,1974).

2.2.2 Taxonomy and history of lupin

Lupin is the seed of Lupinus species in family Fabaceae (or Leguminosae) and sub family Papilionoidea there are four major agricultural important lupin species namely *Lupinus angustifolius, Lupinus albus, Lupinus luteus and Lupinus mutabilis. Lupinus angustifolius* is the most predominantly grown species in Australia and referred as Australian sweet lupin (ASL) or narrow leaf/ blue lupin as well. Australia is the largest lupin producer in the world (FAOSTAT, 2015). Lupin has been used as an ornamental plant in the garden and as an agricultural crop for thousands of years. Also, it has been used as a traditional food in the Mediterranean region and the Andean highlands in South America. In Australia a modern farming system based on wheat lupin rotation has been in place over 40 years (DAFWA, 2012). It has an ancient history in agriculture that traces back more than 4000 years (Kurlovich, 2002). First domestication was occurred in the Mediterranean region and the American continent but the real breakthrough that made lupin a modern agricultural crop occurred in Europe and Australia.

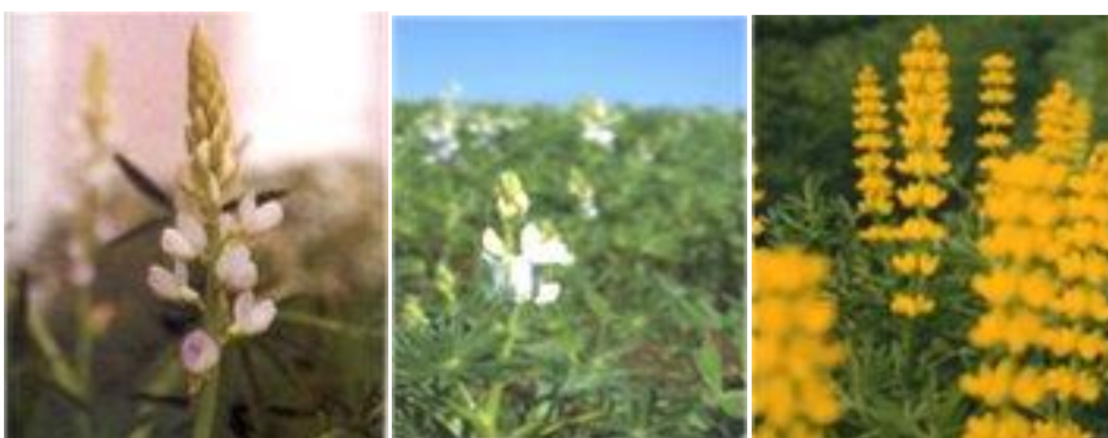


Figure 2.1 Flowers of different lupin species
Source: http//www.public-domain-image

Figure 2.2 Lupin seed
Source: http//www.public-domain-image

2.2.3 Structure of lupin seed

As shown in Figure 2.3 lupin comprises of seed coat (hull) and kernel (cotyledon). The hull fraction of *L. angustifolius* is about 25% of seed weight and values for *L. luteus, L. albus and L. mutabilis are* 30%, 15%, and 12%, respectively. Lupin hull is mostly cellulose and hemicellulose. In contrast the kernel includes cell wall materials/pectin like dietary fibre (30%), protein (40%), fat (5-7%), oligosaccharides (6%), phytic acid (1%), and moisture (12%), respectively. The complete seed including hull fraction and kernel will be referred to as “whole lupin seed” and the kernel portion after removing the hull fraction will be referred to as “dehulled lupin seed”.

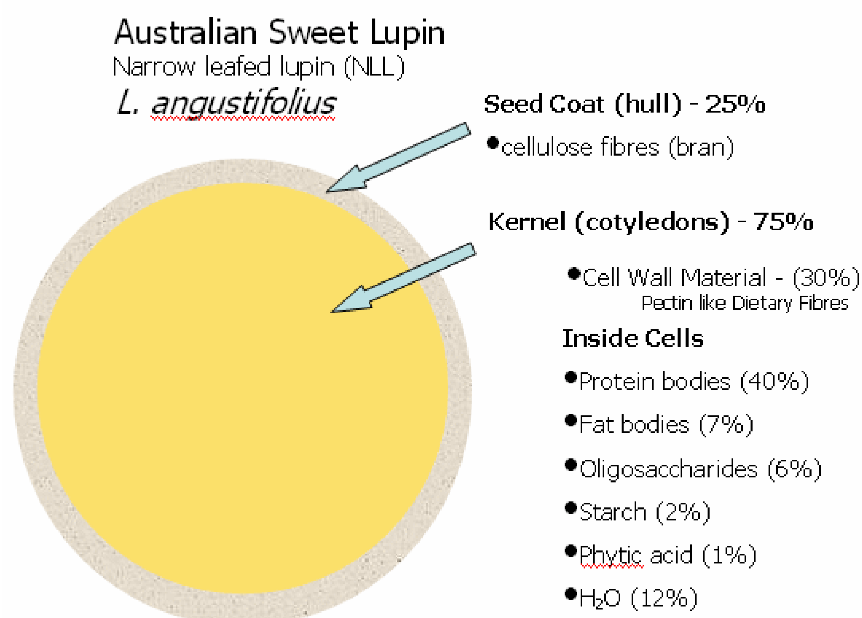


Figure 2.3 Structure of lupin seed.

Source: lupin food.org, http://www.lupins.org/feed

2.2.4 Chemical composition of lupin

Table 2.1 presents the proximate composition of whole lupin seed and dehulled lupin seed and each nutritional component will be explained in detail (McCleary & Prosky, 2008). Contribution of these compounds in lupin compared to some other legumes is shown in Table 2.2.

Table 2.1 Percentage seed coat and proximate composition of whole lupin seed (WS) and de-hulled lupin seed (DHS)

	L. albus		*L. angustifolius*		*L. luteus*		*L. mutabulis*	
Component	WS	DHS	WS	DHS	WS	DHS	WS	DHS
Seed coat (%)	24	0	18	0	27	0	16	0
Moisture (g 100 g-1)	9	12	9	11	9	12	8	10
Protein (g 100 g-1 db)	32	41	36	44	38	52	44	52
Fat (g 100 g-1 db)	6	7	9	11	5	7	14	17
Ash (g 100 g-1 db)	3	3	3	4	3	4	3	4

Source of data: (Mcleary & Proxy 2008)

Table 2.2 Main nutritional components of selected legumes (values in g/100g db)

Component	*Lupinus* spp.	Pea (*Pisum sativum*)	Soybean (*Gycine max)*
Protein	36-48	20-25	31-36
Lipid	4-12	1-2	16-21
CHO (available)	5-8	44-54	5-6
Mineral	3-4	3-4	4-5

Source of data: (Mcleary & Proxy 2008)

CHO- carbohydrate

2.2.4.1 Protein

Lupin kernels contain ~ 42 g 100 g^{-1} db protein, which is close the protein content of soybean but higher than other legumes such as chickpea, lentil and pea (28, 28.6, and 23.3 g 100 g^{-1} db. respectively) (Roy et al., 2010). As in other legumes, lupin does not have a nutritionally balanced amino acid profile due to scarcity of the sulphur containing amino acids, methionine and cysteine (da Silva et al., 2011). Intake of an adequate amount of protein containing balanced essential amino acid composition and good bioavailability is an essential dietary component (Rolfes et al., 2009). Hall et al.(2005b) reported that Australian sweet lupin (ASL) kernels contained 41.8 g 100 g^{-1} db and (Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2016) reported protein values ranging from 39.6-42.2 g 100 g^{-1} db. depending on the variety. Saez, Borquez, Dantagnan, and Hernández (2015) reported that the protein content of *L. albus* whole seed (WS) was 31 g 100 g^{-1} db. and dehulled seed (DHS) 41.6 g 100 g^{-1} db. while Schoeneberger et al.(1983) noted that protein content of *L. mutabilis* (WS) ranged

from 41.4-47.7 g 100 g^{-1} db. Protein content of ASL seeds was reported to be affected by both genotype (i.e. variety) and environment (e.g. year of harvest and geographical area) (Cowling and Tarr, 2004). Lupin contains two classes of proteins, albumins and globulins (Duranti et al., 2008). The globulins are the main seed storage protein and are classified into four families: α-conglutin, β-conglutin, γ-conglutin, and δ-conglutin (Foley et al., 2011). These α, β, γ and δ-conglutins make up of 75% of the total protein in the seed (Duranti et al., 2008). As in most other legumes, the composition of amino acids in lupin seed is not as balanced as that of animal proteins such as in egg protein (Mandal and Mandal, 2000, Sujak et al., 2006, chew et al., 2003). The major limiting essential amino acids in lupin seeds are methionine, cysteine, valine and tryptophan (Doxastakis et al., 2002). The amounts of the other essential amino acids i.e. lysine, isoleucine, leucine, phenylalanine and tyrosine are comparable to the Food and Agricultural Organization (FAO) standards for amino acids of the ideal reference protein appropriate for adults (FAO/WHO/UNU, 1985).

Some studies have indicated that β-conglutin may have a role in reducing serum cholesterol levels (Sirtori et al., 2004). However, a study using proteomic analysis has shown that β-conglutin is the major allergen of *L. angustifolius* (Danica et al., 2008). In soybean its analogue, β-conglycinin was also found to be a food allergen (Krishnan et al., 2009). Further, δ-conglutin is the major sulphur-rich protein found in lupin seeds; therefore, it is a nutritionally important component (Lilley, 1986). It is however thought that the δ-conglutin is also a major allergen in lupins (Monsalve et al., 2004). On the other hand, γ-conglutin has been reported to have the ability to lower blood glucose and therefore, potential health benefits (Duranti et al., 2008). In addition, an *invitro* study showed that a lupin protein isolate that contained high level of γ-conglutin had ACE-inhibitory activity,

antioxidant activity, and bile acid binding properties that may be beneficial (Yoshie-Stark & Wäsche, 2004). Furthermore, Sirtori et al. (2004) reported that γ-conglutin from *L. albus* may have hypocholesterolemic properties by stimulation of LDL receptors as shown in animal studies and HepG2 cell lines. (Magni et al., 2004) found that γ-conglutin extracted from *L. albus* was also able to reduce glucose levels in a hyperglycaemic rat model. Hence, γ-conglutin appears to have a range of beneficial bioactive properties and therefore may contribute to the functional food properties of lupin-based foods.

2.2.4.2 Carbohydrates and dietary fibre

Dietary fibre is defined as the organic compounds of plants which cannot be digested and absorbed in the human intestine. Lupin consists of two main tissues, the seed coat (hull) and the kernel (cotyledons). Dietary fibres of lupin hull and kernel are very different. Lupin hull contains a very high amount of total dietary fibre (TDF) (~ 80 g 100 g^{-1} db) mostly insoluble dietary fibre (Guillon & Champ, 2007). Hull TDF is mainly composed of cellulose, insoluble hemicelluloses and lignin. Other minor constituents of the hull are protein, lipid and ash. Further, a higher proportion of phytochemicals (e.g polyphenols,) and minerals especially Ca are found in the hull fraction rather than the kernel (Hung, Handson, Amenta, Kyle, & Yu, 1988; Dueñas, Hernández, & Estrella, 2006). In contrast, lupin kernel is lower in TDF than the hull and contains less cellulose. However, it contains much more dietary fibre compared to other legumes cotyledons (Dueñas, Hernández, Estrella, & Fernández, 2009; Khan et al., 2015). Table 2.3 shows the amount of dietary fibre of lupin and soybean.

Table 2.3 Concentration of TDF, IDF and SDF (g 100 g^{-1} db.) of lupin (*L. albus*) and soybean (*G. max*)

Component	Lupin *(L. albus)*		Soy *(G. max)*	
	WS	DHS	WS	DHS
TDF	50.4	42.9	35.5	35.9
IDF	48.4	38.8	32.6	26.2
SDF	2.0	4.1	2.9	9.7

TDF, Total dietary fibre; IDF, Insoluble dietary fibre; SDF, soluble dietary fibre; WS, whole seed; DHS, dehulled seed

Source of data: Písaříková and Zralý (2010)

The TDF content of dehulled lupin seed (*L. angustifolius)* has been reported to range from 37.5-42.2 g 100 g^{-1} db and there was no significant difference among different lupin varieties grown in the same season in Western Australia ((Villarino, 2014). Further, it has been reported that, TDF in lupin (*L. angustifolius*) kernel contains 74% IDF and 26% SDF (Hall,, Johnson, Baxter, & Ball, 2005). The typical compositions reported for dietary fibre from lupin, and soybean are shown in Table 2.4.

Table 2.4 Proportion of IDF and of SDF in whole and dehulled seeds of lupin and soy

	L. albus		*G. max*	
Component	WS	DHS	WS	DHS
IDF	96.0	90.4	91.8	73.0
SDF	4.0	9.6	8.2	27.0

TDF, Total dietary fibre; IDF, Insoluble dietary fibre; SDF, soluble dietary fibre; WS, whole seed; DHS, dehulled seed

Source of data: Písaříková and Zralý (2010)

In lupin hulls, the proportion of cellulose, hemicellulose and lignin are 83-87%, 7-10%, and 17%, respectively. While, in the kernel, hemicellulose is the major component (56%), followed by cellulose (36-44%) and lignin (3-8%), (Górecka, Lampart-Szczapa, Janitz, & Sokolowska, 2000). The TDF content in lupin seed is higher than in soybean (Jayasena and Quail, 2004). On the other hand, the starch or digestible polysaccharide content of lupin (whole seed) is less than 1% (White et al., 2002) A current standard method for dietary fibre analysis is “Integrated total dietary fibre (INTDF) method (AOAC method 2009.01 & 2011.25). Using this method, insoluble dietary fibre (IDF), soluble dietary fibre precipitate by 78% ethanol (SDFP) or high molecular weight dietary fibre (HMWDF) and soluble dietary fibre soluble in 78% (SDFS) or low molecular weight dietary fibre (LMWDF) can be measured (AOAC method 2011.25). SDFS fraction in lupin mostly includes oligosaccharides and compared to other legumes, lupin seed contains high amount of raffinose family oligosaccharides (RFOs). The RFOs content of different lupin varies significantly ($P < 0.05$) according to the variety and ranged from 7.6 -16.8 g 100 g^{-1} db. (Karnpanit et al., 2016).

2.2.4.3 Lipids

Lupin (whole seed) has high content of unsaturated fatty acids (UFA) and in *L. albus* the proportions of saturated fatty acid (SFA) and UFA are 12 (g 100 g^{-1} db.) and 88% of total lipid, respectively. Palmitic acid (9%) was the main component of SFA in *L. albus* while among UFA oleic acid (46%) linoleic acid (25%) and linolenic acid (12%) were found respectively (Hamama & Bhardwaj, 2004). Geographical location of the lupin production is also a factor affecting oil content of lupin seeds, for example lupin seeds from a continental location (in terms of Italian climatic regions, locations have been classified as continental and

Mediterranean) had lower oil content (8.40 g 100 g-1 db.) than those from Mediterranean locations (11.08 g 100 g^{-1} db.) (Boschin et al., 2007). *L. mutabilis*, contains higher oil content (17 g 100 g^{-1} db.) than other lupin species (Fleetwood and Hudson, 1982) and the content is comparable with the oil content of soybean (20 g/100g) (Kyle, 1994). In general, lupin has a nutritionally beneficial lipid profile with a high level of mono and polyunsaturated fatty acids and a range of sterols and other bioactive compounds

2.2.4.4 Mineral composition and distribution

Lupins, as other legume seeds, are also good dietary sources of minerals and some remarkable differences have been described in mineral contents either between lupin species or in comparison to other legumes. Lupin seeds have low levels of calcium and phosphorus but are similar or even better sources of trace minerals such as iron, zinc and copper when compared to other legumes (Hill, 1977; Sathe *et al.,* 1984; Donangelo *et al.,* 1986). Another striking observation is the very high level of manganese found in some varieties of *L. albus* (up to 350 mg 100 g^{-1}) in comparison to other species (less than 4 mg 100 $g^{-1)}$ (Gladstones & Driver, 1962; Burbano *et al.,* 1982; Savage *et al.,* 1988). Mineral compositions of lupin seeds have been measured mainly in cultivars (Hill, 1977; Burbano *et al.,* 1982; Savage *et al.,* 1988) but little is known about mineral composition of wild lupin species. The biological utilization of dietary minerals is dependent upon several factors including antinutritional components such as phytate which may adversely affect mineral absorption (Santstrom,1988). Therefore, the mineral content of a food product or diet should be determined together with its phytate content, in order to have a better evaluation of its potential as a mineral source. With the exception of calcium and aluminum in both species and boron in *L albus*,

minerals were present at a much higher concentration in the endosperm than in the hull. High concentrations of nitrogen and sulphur in the endosperm of both species reflect the fact that lupin seed protein and sulphur-containing amino acids were stored mainly in the endosperm. All elements studied except sulphur, phosphorus and iron were present at much lower concentrations in lupin protein isolate than in spray dried lupin extract. The mineral composition of lupin protein isolate prepared from both species can be compared favorably with that of soya bean protein isolate. The results of this study indicate a potential for replacing soya protein isolate with lupin protein isolate.

Table 2.5 Mineral composition of *L. angustifolius* and *L. albus* (values in g kg^{-1} or mg kg^{-1} db)

Mineral	*L. albus*			*L. angustifolius*		
	Seed	Hull	endosperm	Seed	Hull	Endosperm
N(g $kg^{-1)}$	58	5.8	72	51	5.2	72
P(g kg^{-1})	4	0.3	5.4	2.4	0.3	4.2
K(g kg-^{1})	9.5	5.4	11.6	8.1	4.6	7.8
Ca(g kg^{-1})	1.9	6.5	1.0	2.4	6.9	1.2
Mg(g kg^{-1})	1.4	0.8	1.8	1.6	1.3	2.0
Fe(mg $kg^{-1)}$	43	28	39	52	27	52
Zn(mg $kg^{-1)}$	38	9	49	35	26	39
Cu(mg $kg^{-1)}$	7	4	8	4	3	6
Mn(mg $kg^{-1)}$	1316	268	1370	61	43	74

Source of data: Hung et al., (1988)

2.2.4.5 Phytochemicals

Phytochemicals are an important group of plant-derived compounds which is responsible for functional food effect (disease protection ability) of plant foods i.e tea, wine, fruits, vegetables, and cereals. Phytochemicals are classified into different groups based on their chemical structure, namely polyphenols, phytoestrogens, terpenoids, carotenoids, phytosterols and phytohemagglutinins (Khan et al., 2016). Phytochemicals are a large group of plant-derived compounds assumed to be responsible for much of the disease protection conferred from diets rich in fruits, vegetables, beans, cereals, and plant-based beverages such as tea and wine. Based on the chemical structure, they are classified into various groups, for instance, polyphenols, phytoestrogens, terpenoids, carotenoids, limonoids, phytosterols and phytohemagglutinins (Rochfort & Panozzo, 2007). Nowadays, scientific community is not only relying on the protein contents of legume crops, but their phytochemical composition is considered equally important to consume them as food and feed. Lupin seeds have significant amounts of phytochemicals specially polyphenols, phytosterols and squalene (triterpene) in comparison with other legume crops (Table 1) (Kalogeropoulos et al., 2010). Although soya bean contains higher amounts of isoflavones (Rochfort & Panozzo, 2007) and tocopherols (Boschin & Arnoldi, 2011), lupin can be an ingredient of choice by the food industry because of its overall nutritional profile and lower price. Polyphenols Work on the identification of phenolic compounds in lupin has been performed only in few species. Understanding of the structural diversity of total phenolics is of great interest to demonstrate various related biological activities. Generally, the major phenolic compounds identified in lupin species belong to subclass flavones, phenolic acids and isoflavones (Fig. 1 and Table 2). For instance, in L. angustifolius seeds, the

flavones, phenolic acids and isoflavones are present in 76%, 19% and 4% of the total identified phenolics, respectively (Duenas et al., 2009). The main identified flavones in the group are aglycone and/or glycosides of luteolin, apigenin and diosmetin, while the principal contribution in the isoflavone group is from genistein and its derivatives. In phenolic acids, protocatechuic acid (hydroxycinnamic acid) and p-hydroxybenzoic acid are the major representative components (Siger et al., 2012). Although flavones are found in higher quantities, many of the isoflavones present in lupin species get more importance because of their nonsteroidal phytoestrogenic activity in mammals (Ranilla et al., 2009). In addition, similar to flavones, they are good antioxidants because of their ability to trap singlet oxygen. According to the published information, the first identification of isoflavone in lupin species was reported by Fukui et al. (1973) in immature seeds of L. luteus. Phenolic compounds can be found in all parts of lupin plant, that is stem, leaves, roots and seeds, and their concentrations vary depending on the plant part. A study on *L. exaltatus* has shown higher contents of isoflavones and their conjugates in roots than in stems, while free aglycones were identified in roots and inflorescences (Garcia-Lopez et al., 2006). Variations of lupin species in phenolic contents have been observed among different cultivars and growth locations.

Genistein and daidzein derivatives

Legumes are considered as major sources of isoflavone derivatives genistein and daidzein. Kaufman, Duke, Brielmann, Boik, & Hoyt (1997) claimed that lupin, fava bean, soybeans, kudzu and psoralea contain high amount of genistein and daidzein. Genistein is a potential anticancer agent that inhibits platelet aggregation, induce apoptosis, inhibits angiogenesis reduces the bioavailability

of sex hormone and inhibits differentiation of cancer cells (Kuo, Wu, & Lee, 2012). Daidzein act as an anticancer agent by inducing differentiating in B16 melanoma and HL-60 human leukaemia cells. To produce aglycosides (daidzein and genistein) effectively a process of isoflavone hydrolysis by B. subtilis nattoNTU-18 has used by (Kuo, Wu, & Lee, 2012). In addition, (Choi, Kim, & Rhee, 2002) examined the hydrolysis of soybean isoflavone glucosides by lactic acid bacteria and found that 70-80% the genistin was converted to genistein and 25-40% of the daidzein after 24 h of fermentation.

2.2.5 Health benefits of lupin dietary fibre

Findings from some animal and human studies, have pointed to lupin dietary fibre has having an important role in appetite suppression (Lee et al., 2006b), blood glucose level reduction (Johnson et al., 2003; Magni et al., 2004,), reduction of cardiovascular disease risks (Sirtori et al., 2004, Hall et al., 2005; Martin et al., 2005, Bettzieche et al., 2008) and improvement of bowel health (Johnson et al., 2006, Smith et al., 2006). Due to relatively higher level of oligosaccharide (6.0%) such as raffinose, flatulence is noted as a problem associated with consumption of lupin, but this can be overcome by fermentation of lupin as these oligosaccharides are removed by this process (Refstie, Sahlström, Bråthen, Baeverfjord, & Krogedal, 2005).

It was found that there is a link between dietary fibre consumption and the prevention of various diseases and disorders (Burkitt, Walker, & Painter, 1974). The beneficial effect of dietary fibre on the chronic diseases has been known for long time, and dietary fibre supplementation has been suggested as a remedy to be used to improve these disease conditions such as hypercholesterolemia, hypertriglyceridemia, hyperglycaemia, obesity and appetite. Lee et al. (2009) found that consumption of bread enriched with lupin flour reduced systolic blood

pressure in overweight men and women. Furthermore, it has also been found that the replacement of dietary carbohydrates by protein- and fibre-enriched lupin foods also reduced arterial blood pressure (Belski, 2012).

2.2.6 Lupin and health benefits

The increasing trend of non-communicable diseases linked to metabolic syndrome (MetS), including type-2 diabetes, cardiovascular disease as well as cancers are major public health issues in both developed and developing countries (Baldeón, 2012). The majority of these are diet and nutrition related. As a result, there is a growing interest to develop novel foods or ingredients to assist with their management or treatment. A range of lupin-based novel foods such as noodles (Jayasena, Leung & Nasar-Abbas, 2010), tofu (Jayasena, Khu & Nasar-Abbas, 2008) pasta (Jayasena & Nasar-Abbas, 2011) and tempeh (Jayasena, 2006) have been developed. Lupin can be used as a low cost functional food ingredient (Magni et al., 2004; Hall, Thomas, & Johnson, 2005; Sirtori et al., 2009; Baldeón, 2012). There are only few studies available on effect of fermentation on lupin using specific bacteria which demonstrated decreased sucrose, phytates and flatulence occurrence oligosaccharides (Camacho et al., 1991). However, Bacillus fermentation of lupin reduced vitamin C and vitamin E activity while total phenolic compounds increased (Camacho et al., 1991; Frias et al., 2005; Fernandez-Orozco et al., 2008).

Lupin has been mainly used as an animal food for many years, but recently there is a growing interest for consumption of lupin and lupin-based products as functional foods in particular for blood glucose control (Magni et al., 2004; Bertoglio et al., 2011; Rosa Lovati et al., 2012). Also, fermented legumes have been used as health food in Asia for long period of time and these fermented legumes have shown blood-glucose modulatory effects (Taniguchi et al., 2008;

Kwon, Daily, Kim, & Park, 2010; Taniguchi-Fukatsu et al., 2012). Consumption of Lupin has shown many health beneficial effects and fermentation would be expected to give a fermented lupin product with better health beneficial effects (Kwon et al., 2010). There are some evidence research findings to support hypoglycaemic activity of lupin. Oral administration of lupin ɣ-conglutin on the diabetic rats significantly reduced blood glucose level compared to metformin, a diabetic medication (Magni et al., 2004). Potential therapeutic use of lupin protein has been noted in mouse myoblast and insulin mimetic action of ɣ-conglutin has been demonstrated as biochemical pathway of the reaction (Terruzzi et al., 2011). Moreover, after oral treatment of γ-conglutin, decreased blood glucose levels in hyper-glycaemic rats have been noted (Rosa Lovati et al., 2012) Similarly, a lupin seed γ-conglutin enriched preparation has resulted dose dependent decrease of blood sugar in both healthy human subjects and experimental animals (Bertoglio et al., 2011). Adding Australian sweet lupin flour into wheat flour (white) in bread making has been demonstrated to lower glycaemic index of the bread without affecting palatability (Hall et al., 2005).

Similar to the epidemic level of elevated blood sugar problem all over the world, elevated serum lipids associated with CVD is also one of the major public health issue in the world at present (Weisse et al., 2010). Substitution of plant protein in the diet of laboratory animals with high cholesterol have demonstrated reduced cholesterol. According to the US Food and Drug Administration (FDA, 1999) the consumption 25 g of soy protein per day reduces cholesterol in subjects with elevated blood cholesterol. Lupin protein isolates and whole lupin have shown protective effects on hepatic steatosis (fat accumulation in the liver) because of their hypocholesterolemic and hypo-triglycereamic effects (Marchesi, 2008; (Osman et al., 2011; Fontanari, Batistuti, Cruz, Saldiva, & Arêas, 2012) Similarly,

protein of white lupin seed reduced LDL cholesterolemia in rats. Lupin protein reduces LDL cholesterol and LDL: HDL ratio (Hall et al., 2005; Bähr et al., 2013). Lupin protein isolated from *L. albus* demonstrated hypo-triglyceridaemic effect in rats (Spielmann et al., 2007). Lupin and soya attenuate blood pressure and this positive effect is independent of lipid profile or blood glucose level (Pilvi, 2006). Regular consumption of lupin enriched bread reduced hypertension (Yang et al., 2010). Belski et al., (2011) found that lupin enriched food plays a role for improving CVD health. Research reveals that lupin enriched foods, which are naturally high in protein and fibre have significant positive effect on CVD risk factors (Hall, Johnson, Baxter, & Ball, 2005). Animal experiment conducted by (Mousa, 2015) indicated that diet supplemented with different functional sweet lupin biscuits significantly reduced serum total cholesterol, triglycerides, low density lipoprotein, ratio of low density/high density lipoproteins.

Hodgson et al. (2010) noted that a diet higher in protein and fibre derived from lupin-enriched food does not have any effect on weight loss of overweight or obese people. However, such a diet provides cardiovascular health benefits in terms of insulin sensitivity and blood pressure. Lee et al. (2009) showed that increasing protein and fibre in bread with lupin flour could be a simple dietary approach to help reduce blood pressure and CVD risk.

Another important health benefit of lupin is the satiety control effect. According to (Lee, 2006) a new food, enriched in protein and fibre derived from lupin flour significantly influences energy intake. In addition, the first demonstration of human appetite suppressing behaviour of lupin fibre was reported by Archer, Johnson, Devereux, & Baxter (2004).

Sumi et al. (1987) identified and isolated a potent fibrinolytic enzyme nattokinase (NK) from the traditional Japanese fermented natto. They confirmed that oral administration of NK or natto produced a mild frequent enhancement of the fibrinolytic in the plasma, as indicated by the fibrinolytic parameters and the production of tissue plasminogen activator. Chang, Fan, Kuo, & Sung (2000) and Fujita et al. (1993) indicated that NK is a subtiline like serine protease. Serine proteases are enzymes that cleave peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the (enzyme's) active site (Hedstrom, 2002). Serine proteases fall into two broad categories based on their structure: chymotrypsin-like (trypsin-like) or subtilisin-like (Madala, Tyndall, Nall and Fairlie, 2010). In humans, they are responsible for coordinating various physiological functions, including digestion, immune response, blood coagulation and reproduction. The enzyme is composed of 277 amino acids with a molecular weight of 27.7 KDa. Natto has a history of 2000 year and traditionally it was used as a folk medicine for heart and vascular disease, to relieve fatigue and as an anti-beriberi agent (Fujita et al., 1993). Ero, Ng, Mihailovski, Harvey, and Lewis (2013) showed that nattokinase can be measured directly in the blood of humans following ingestion of a commercial preparation. At present, there are many preparations of nattokinase marketed all over the world including United States and Japan as over the counter dietary supplement. Furthermore, one study conducted in Taiwan showed that nattokinase decreases plasma level of fibrinogen factor Vii and factor viii which means supplementation of nattokinase is beneficial for human with risk factors of CVD. They also indicated that administration of nattokinase is safe in healthy volunteers, patients with CVD risk factors and dialysis patients (Chang, Fan, Kuo, & Sung, 2000).

Another important research has been reported on amyloid β (Aβ) degrading ability of nattokinase from *B. subtilis* (Hsu et al, 2009). Aβ plaque formation is one of a pathological characteristic of Alzheimer's disease (AD) and Aβ 40 is a major product of amyloid precursor protein (APP). According to an amyloid degrading test, nattokinase dissolved amyloid fibrils (Hsu, Lee, Wang, Lee, & Chen, 2008; Hsu et al, 2009). However, it is necessary to conduct more studies to evaluate the therapeutic activity nattokinase. Since natto has been used as a human food for long time, it would be beneficial to conduct an epidemiological study on rate of occurrence of having Aβ related Alzheimer's disease in a population regularly consuming natto.

Angiotensin-converting-enzyme (ACE) inhibitory peptides are formed by breaking down of protease enzyme nattokinase and they are considered as vasodilator. *Bacillus subtilis natto* fermented soybean products have shown the ACE-inhibitory activity and the antihypertensive activity (Fernandez-Orozco et al., 2007). Similarly, B. *subtilis* fermented soybean meal (Wang, Zha ng, Sun, & Dai, 2013), and *B. subtilis* fermented hydrolates of soybean proteins (Yoshie-Stark & Wäsche, 2004) showed the ACE-inhibitory activity and the antihypertensive activity.

Phytic acid (PA) or myo-inositol hexakis phosphates are the main storage form of phosphorus in many cereal and legume seeds. Phytic acid is considered as an anti-nutritional factor because it forms insoluble complexes with minerals such as calcium, magnesium and iron, reducing their bioavailability due to its strong chelating property. Fermentation has been used to reduce or remove phytic acid from the food to improve bioavailability of minerals. Phytase is responsible for enzymatic degradation of phytic acid and it hydrolyses PA into myo-inositol and inorganic phosphates (Da Silva, Trugo, da Costa, Terzi, & Couri, 2011). In solid

state fermentation (SSF) *Aspergillus* (Da Silva et al. 2011) in *lactobacillus* with lupin (Camacho et al., 1991) and in natural fermentation of black gram (Yadav &Khetarpaul, 1994) have been reported to produce phytase enzyme.

2.3 Fermentation

Fermented foods are the products, forms due to action of microorganisms or enzymes produced by them and also, they do desirable biochemical changes and significant modification to the food (Campbell-Platt, 1994). Louis Pasteur defined fermentation as "La vie san I, air" or "life without air" (Bourdichon, et al, 2012). Also, it is defined as chemical transformation of complex compounds into simple compounds by the action of enzymes, organic catalysts produced by microbes such as bacteria, mould and yeasts (Corma, Ibora, & Velty, 2007). Fermentation is well known for improving the nutritional value of legumes and their sensory acceptability. In addition, fermentation can eliminate certain undesirable compounds such as phytic acid and some anti-nutritional factors and make some bioactive compounds such as peptides more accessible thus increase bioavailability(Deshpande, 2000).Fermentation is an important method of food preservation by suppressing growth of pathogenic organisms and changing pH by producing acids and the action of the fermenting bacteria, fungi and mould can enhance the nutritional quality of the product (Fernandez-Orozco et al., 2007). Fermentation can also decrease cooking time, detoxify and enhance flavour, aroma and texture (Deshpande, 2000).

The major effect of fermentation is the hydrolysis of complex macronutrients such as carbohydrates, protein and lipids into simpler forms that can enhance their bioavailability. For instance, in tempeh, although it's proximate composition does not change during fermentation, percentage protein content increases due to

reduction of carbohydrate as microorganism uses them for their metabolism (Stanton, 1993). Legumes in general, are deficient in the essential sulphur containing amino acids methionine and cysteine, while lack of lysine is a major nutritional issue in cereal grains. This mixed cereal and legume fermentation is a great opportunity for complementing amino acid content. It has been reported that during the process of tempeh fermentation methionine content increased and in another Indian traditional fermented legume and rice food fermentation process lysine content increased (Hermansen, Sondergaard, Hoie, Carstensen, & Brock, 2001). Moreover, traditional fermentation increases vitamin content in some fermented foods (Liem, Steinkraus, & Cronk, 1977). Although fermentation improves the nutrition profile of many substrates there are situations which lead to losses of nutrients. Over fermenting or excessive fermentation is such kind of situation and it gives rise to proteolysis of substrate, forms ammonia and reduces protein content of the product (Deshpande, 2000). Also washing and soaking may reduce water soluble minerals and other nutrients. Legume fermentation with *B. subtilis* can be seen in many countries whereas natto in Japan, Thua-nao in Thailand, Chung-kook-jong in Korea and Kinema in Nepal are among them (Sarkar, Cook, & Owens, 1993).

Different fermented foods including grains/cereals, dairy and beverages are used in diets around the world. These foods contribute to a significant part of all diets worldwide typically about one third of food intake providing a major contribution nutritionally and flavour and interest in our food consumption. This includes direct use of things like mushrooms (Campbell-Platt, 1994).

The history of food fermentation is gone back to antiquity. Beer brewing and wine making in Egypt and Mesopotamia has been recorded from 6000-2000 BC

(Stanton, 1993). Also records of consumption of mushrooms in China date back to 6000 years (Campbell-Platt, 1994). Further, bread making records of Egyptians dates back to 4000 BC. Evidence of fermented milk in India dates back to 2000 BC while oriental fermented foods such as soy source and miso records goes back to several millennia (Stanton, 1993). Therefore, history of fermentation has a very long history and it is one of the oldest known uses of biotechnology. Since then it has been an emerging science and important subject in the modern world with its broadening technology.

Fermented foods comprise vast diverse range of products different regions of the world. (Campbell-Platt, 1987) recorded around 250 types of fermented food products with more than 1000 types of cheeses and over 3500 other individual products. Varieties of milk have been used with the fermentation to produce cheese, yoghurt, curd and kefir which was made using yeast alone or in combination with lactic acid bacteria. It was recorded that, there being over 900 know varieties of cheeses more than 200 types of fermented meats and also more than 200 bread products. Based on substrate used for fermentation, there are eight main groups of fermented foods i.e. cereals, dairy, fruits and vegetables, legumes, meats, roots crops, sea-foods (Table 2. 1). Although Europe is the major region for production of fermented foods such as dairy products, cereals and alcoholic beverages and meats, other food from starch crops legumes and sea-foods are produced primarily in developing countries (Stanton, 1993; Reddy et al., 1986).

2.3.1 The benefits of fermentation as a food processing method

There are many benefits of food fermentation and one of the most important benefits from fermentation of foods is that it is a cheap and energy efficient form of preservation. Fermentation makes food products more palatable by boosting the flavour through the conversion of sugars into acids. Further, fermenting increase grain digestibility and porridge that has been fermented hydrolyses starch into shorter chains of glucose and dextrose. Fermented products often contain higher levels of vitamins especially B- complex vitamins thiamine, nicotinic acid, biotin and riboflavin and proteins. Examples are the Indian legume –cereal mix fermented food called idli, sorghum beer from southern Africa and palm wine from West Africa. Some fermented products such as tempeh, natto and fish source have meat-like flavours and odours which is important for cultures where meat is scarce. Food such as cassava contains semi-dangerous level of cyanide and fermentation can reduce toxicity, making them safe to eat. Fermented foods often contain a higher level of convertible energy than non-fermented foods of the same weight. Some fermented foods have medical advantages associated with a constant intake Eg. Koumiss has found to be effective to control tuberculosis and alimentary tract diseases (Trojanowska, 2006).

2.4 Tempeh

Tempeh is a traditional Indonesian fermented food which is produced from de-hulled soybean seed fermented with the mould *R. oligosporus* (Bavia et al., 2012). As shown in Figure 2.2 (preparation protocol) seeds are soaked in an excess (3 volumes) of water for 18 h at room temperature (25 °C). White vinegar

(acetic acid, pH 4) is then added to promote acidic fermentation (Starzyńska Janiszewska et al., 2014). After subsequent washing with water, the seeds are boiled for 1 h, drained and cooled to 37 °C. Then cooled seeds are inoculated with *R. oligosporus* starter culture (Berghofer, Mundigler, Sentall, & Walcak, 1998) Tempeh is used as a condiment or side dish and used in soups or deep fried before consuming. It is also consumed with staple foods such as rice, corn or cassava where it contributes around 10% of the total protein consumed in Indonesia where it is the major protein source of the country (Karyadi & Widjaja, 1996). Due to concerns of consumers about cholesterol related diseases in developed countries and difficulty in affording to consume animal protein in developing countries, there is an increasing need of protein of plant origin. Tempeh is a protein rich vegetarian food, which is one of the world's first meat analogues and is a staple food in Indonesia which is highly nutritious and low cost. The white cake appearance of tempeh is formed by mould mycelia. During tempeh formation protein, fat, oligosaccharides and phytic acid are hydrolysed to their simpler forms (Handoyo & Morita, 2006).

2.4.1 Health benefits of tempeh

Tempeh has a wide range of reported health benefits such as prevention of non-communicable diseases CVDs and cancers, elimination of bowel diseases such as flatulence and diarrhoea, favourable effects on bone health and promote menopausal health De Mejia & De Lumen, 2006; (Astuti, Meliala, Dalais, & Wahlqvist, 2000) (Kwon et al., 2010) Tempeh was found to contain the higher amount of total isoflavone (daidzein and genistein) compared to other soya products. Raw tempeh contained 26 ± 6 mg daidzein (Da) and 28 ± 11 mg genistein (Ge) while fried tempeh contained 35 ± 11 mg Da and 31 ± 11 mg Ge in 100 g (wet basis). Total isoflavone content in 100 g of raw tempeh, based on

a dry weight, was 205 ± 56 mg and significantly reduced to 113 ± 41 mg in 100 g of fried tempeh. Tempeh in batter was deep-fried for 30 min which reduced 45% of the total isoflavone content compared to the raw one. Raw tempeh contained the highest total content of isoflavone among the studied local soy products. Total isoflavone content in processed soy foods like egg tofu and home-made soybean drink were significantly lower than other soy products studied (Haron, Ismail, Azlan, Shahar, & Peng, 2009). These compounds are similar to estrogen and previous research indicates that women can benefit from their intake to reduce the symptoms of menopause (Haron, Ismail, Azlan, Shahar, & Peng, 2009). Murooka & Yamshita (2008) demonstrated that there is an anti-hypertensive effect of tempeh due to the activity of gamma-amino butyric acid GABA. Furthermore, these isoflavones have demonstrated angiogenesis and endothelial cell proliferation inhibitory properties (Kiriakidis et al., 2005). Mould Fermented tempeh decreased flatulence over baseline values and caused significant delay in the time of gas formation (Calloway, Hickey, & Murphy, 1971). Karmini (1987) studied the effect of tempeh on the control of enteropathogenic diarrhoea in a rabbit model. According to her findings, tempeh fed group of rabbits showed reduced number of animal with diarrhoea symptoms compared to group without tempeh. The effect of tempeh on iron bioavailability and lipid peroxidation in anaemic rats have been studied by (Kasaoka, Astuti, Uehara, Suzuki, & Goto, 1997) and have confirmed that tempeh increased liver iron compared to unfermented soybean without promoting lipid peroxidation. Iron fortification in tempeh showed high iron bioavailability in a study on Wister rats and these animals showed significant increase of haemoglobin levels (Sudargo et al., 2013). According to Spector, (2010) many recent studies are demonstrated the health benefits of tempeh. Hassan et al.(2014) reported on an experiment to test

hypocholesteremic effect of soybean and sweet lupin tempeh in hypercholesteremic rats. They reported that there is a significant reduction of total cholesterol, LDL- cholesterol and triglycerides and favourable increase in serum HDL- cholesterol concentration compared to control (mice fed without soybean and lupin).

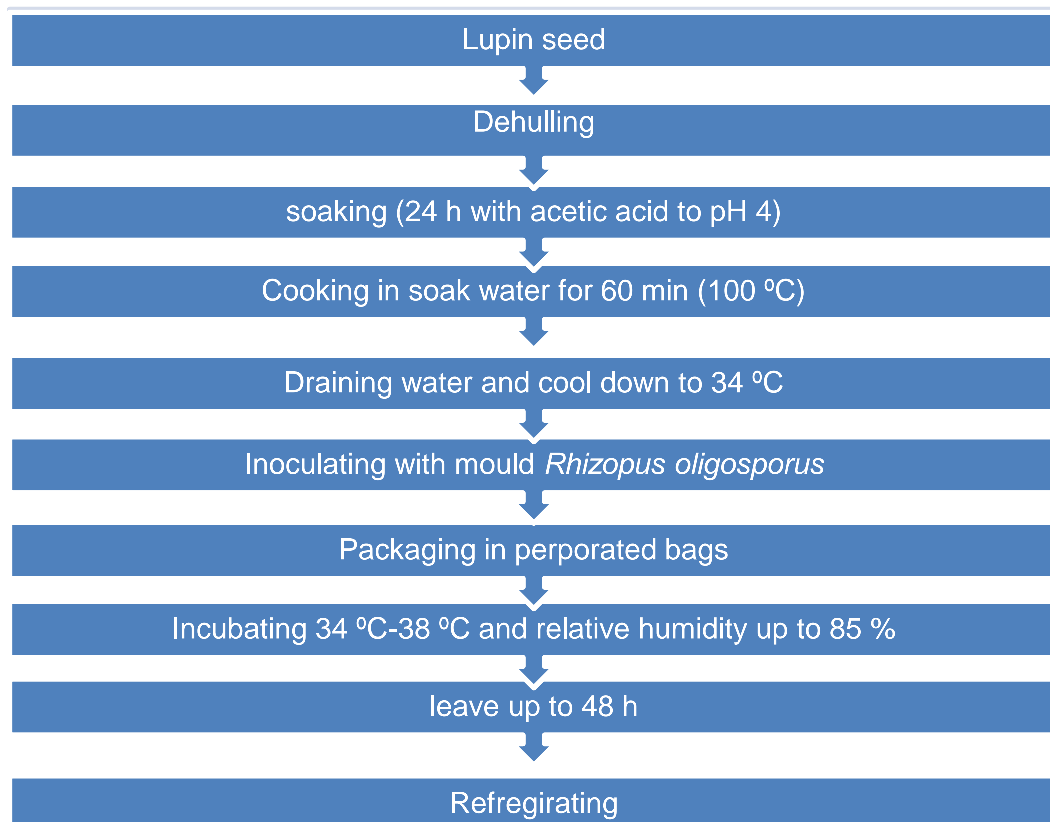


Figure 2.2 Flowchart- preparation of tempeh (Karyadi & Widjaja, 1996)

2.5 Natto

Figure 2.3 shows the protocol of preparation of natto (Deshpande, 2000). Natto is a traditional food from Japan made from boiled whole soybean fermented for about 24 h with *Bacillus subtilis* (Sumi, Hamada, Tsushima, Mihara, & Muraki, 1987b). It has unique flavour, odour and viscous texture. Also, it is low cost, highly

nutritious and has long shelf-life (Ueda, Kubo, Miyatake, & Nakamura, 2007). In the fermentation process, cleaned seeds are soaked overnight and boiled for 20-30 min followed by inoculation with *B. subtilis* at 34-37 °C. Natto is mostly eaten with rice as a side dish using chopsticks and also is used as an ingredient in miso a Japanese traditional soup. Natto originated in northern Japan around 1000 years ago. It is produced by many households in Japan using simple procedures (Hu et al., 2010). The natto market is relatively small compared to other fermented soybean food and it is not that popular internationally, due to unusual aroma, flavour and texture (Yoshikawa, Chen, Zhang, Scaboo, & Orazaly, 2014).

During industrial natto preparation, soybean seeds are soaked in a large tank to soften them. The temperature of the water tank should be about 12 °C, and the seeds are soaked for 20 h. Appropriate soaking time is required to optimise the texture and taste of the natto. In order to get a high quality natto product, the soaking tank must be cleaned regularly. It is particularly important to remove lactic acid, since it inhibits the growth of *Bacillus subtilis natto* and impairs fermentation. Small-seeds weighing around 70 mg are usually used for natto production. Preferably seeds that are about 5 mm in diameter and have a clear or yellow hilum colour and a uniform round shape are used. Small-seeds have some desirable characteristics for high quality natto production such as rapid water absorption, high carbohydrate content, low calcium content, and low oil content (Chen et al., 1993; Wei and Chang, 2004). Amylase and protease, which contribute to the softness and unique taste of natto, are produced by the bacteria that grow on the surface of the soybeans (Kiuchi, 2008). The rehydrated seeds are then pressure cooked and 1.5 kg cm $^{-2}$ of pressure is applied at 120 °C for

20 min to the soaked seeds to soften them. Undesirable bacteria are killed during the steaming process, however *B. subtilis natto* is able to survive the high temperature. Then, seeds are immediately inoculated with a *B. subtilis natto* spore suspension, while the cooked seeds are still around 85 °C. If the cooked soybeans are inoculated after cooling, there is a greater risk that they will be contaminated with other undesirable bacteria species. The concentration of *B. subtilis natto* in the soybean stock, about 10 colonies forming units (CFU) per gram of soybeans, is critical for inoculation as too high a concentration inhibits the development of the desired stickiness. After adding the spore suspension,
50 g of natto soybeans are packed in small polystyrene or paper cups or containers. During fermentation step, the packed natto are placed in the fermentation room for about 24 h. Oxygen, temperature, humidity, and time are properly adjusted. During the first 1 to 6 h of fermentation, 85 to 90% humidity at 40 °C is needed. In the second stage, between 6 and 16 h, 75% humidity is required. During the third stage, between 16 and 24 h, 55% humidity is required. After 8 h of fermentation, stickiness is produced. At 18 to 20 h, temperature is lowered to 10 °C then after 4 to 6 h and natto packages are moved to a refrigerator with a temperature set at 5 °C to control the fermentation of *B. subtilis natto* for three days to produce the final product.

During natto fermentation, many complex biochemical changes occur such as secretion of proteases on the outer layer of soybeans, incorporation of hydrolysed amino acids, racemisation of L-glutamic acid into the D form, and formation of poly-ɣ-glutamic acid (ɣ-PGA) (Morinaga et al., 2006). Natto and nattokinase which is active ingredient extracted from natto, have been well known for functional food effect thereby improving health by reducing blood cholesterol

levels, decreasing blood pressure and inhibiting osteoporosis. Therefore, natto is considered as a popular functional food worldwide (Milner & Makise, 2002).

Natto also contains antihypertensive substance compounds such as ACE-inhibitory peptides (Okamoto, Hanagata, Kawamura, & Yanagida). Soybean fermented by *B. natto* has potential to become a functional food because of its high antioxidant action (Hu et al., 2010). A Japanese meal which contained natto and a viscous vegetable, suppressed postprandial glucose and insulinemia in healthy subjects. Therefore, this dietary combination leads to reduce risk of diabetes and cardiovascular disease (Taniguchi et al., 2008). A study conducted on overweight subjects with impaired glucose tolerance showed, improved insulin sensitivity, serum lipids and oxidative stress on natto consumption (Taniguchi-Fukatsu et al., 2012). Fujita et al. (1993) indicated that nattokinase, available in natto can be used as a natural agent for oral fibrinolytic therapy.

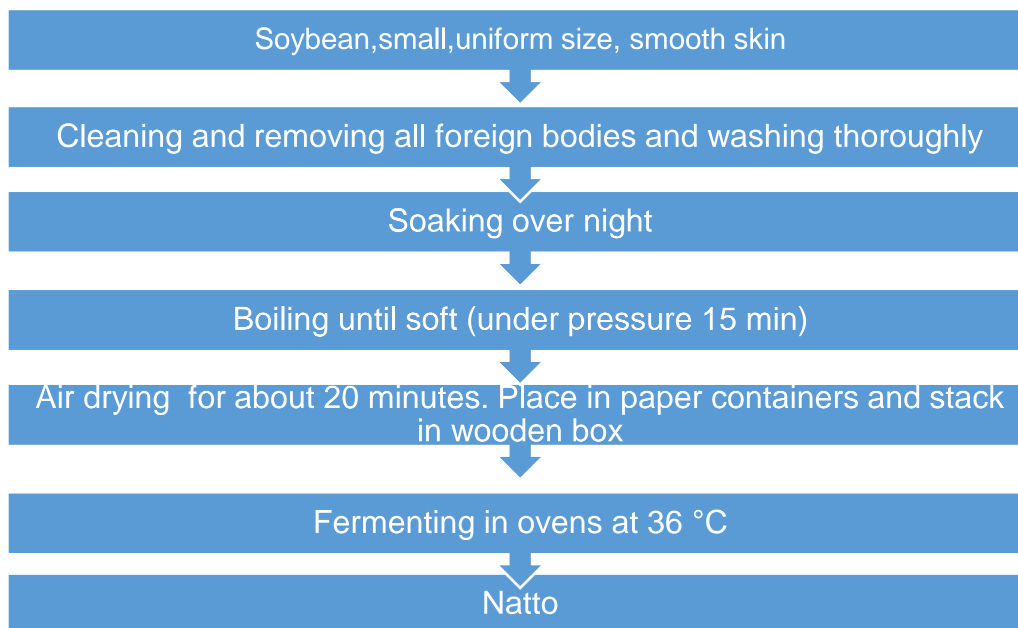


Figure 2.5 Flowchart - preparation of natto (Deshpande, 2000)

2.5.1 Sensory properties of natto

In a natto sensory evaluation test the most import attributes to the consumers were natto flavour, followed by hardness, smell, and stickiness (Matsunaga et al., 2001). In addition, they were also concerned about food safety, such as pesticide use, organic soybeans, and non-GM soybeans. In general, natto made from small-seeded soybeans has a strong flavour and sweetness, while natto from large-seeded soybeans has less flavour and taste. High sugar content should be good for the final natto product as well as for its stickiness and sweetness (Muramatsu et al., 1997). Wei and Chang (2004) used 10 sensory panellists at North Dakota State University and the panellists tested a commercial natto product, itobuki natto, and natto made from four other natto soybean lines: Danatto, Minnatto, Natto King, and MN91-468. The sensory attributes evaluated were natto aroma, colour, hardness, stickiness, bitterness, ammonia flavour, sweetness, sourness, and chewiness. The unstructured scale evaluation provided to panellists included colour (light and dark), aroma (weak and strong), hardness (soft and hard), stickiness (not sticky and very sticky), bitterness (not

bitter and very bitter), ammonia flavour (weak and strong), sweetness (not sweet and very sweet), sourness (not sour and very sour), and chewiness (not chewy and very chewy). They concluded that hardness and stickiness were the most important properties of natto, and natto hardness is positively correlated with chewiness. Also, there is a correlation between chewiness and natto processing conditions and natto cultivars. Even though natto breeding has been conducted at several universities and food industries, studies on sensory analysis are very limited.

In a natto sensory evaluation test with, 400 Japanese consumers Matsunaga et al. (2001) found that consumers were concerned in natto flavour, followed by hardness, smell, and stickiness. In addition, they were also curious about food safety, such as pesticide use, organic soybeans, and non-GM soybeans. In general, natto made from small-seeded soybeans has a strong flavour and sweetness, while natto from large-seeded soybeans has less flavour and taste. High sugar content should be good for the final natto product as well as for its stickiness and sweetness (Muramatsu et al., 1997). Wei and Chang (2004) used 10 sensory panellists at North Dakota State University and the panellists tested a commercial natto product, itobuki natto, and natto made from four other natto soybean lines: Danatto, Minnatto, Natto King, and MN91-468. The sensory attributes evaluated were natto aroma, colour, hardness, stickiness, bitterness, ammonia flavour, sweetness, sourness, and chewiness. The unstructured scale evaluation provided to panellists included colour (light and dark), aroma (weak and strong), hardness (soft and hard), stickiness (not sticky and very sticky), bitterness (not bitter and very bitter), ammonia flavour (weak and strong), sweetness (not sweet and very sweet), sourness (not sour and very sour), and chewiness (not chewy and very chewy). They concluded that hardness and

stickiness were the most important properties of natto, and natto hardness is positively correlated with chewiness (r = 0.73). Also, there is a correlation between chewiness and natto processing conditions and natto cultivars. Even though natto breeding has been conducted at several universities and food industries, studies on sensory analysis are very limited.

2.5.2 Health benefits of natto

Natto has been known as a functional food for more than the 1000 years (Sumi, Ikeda, & Ohsugi, 2009). It has many health benefits which are attributed to different bioactive compounds per se and one such compound is nattokinase, an extracellular fibrinolysin (Sumi et al., 1986). Nattokinase has higher fibrinlolytic activity than other similar agents while dietary natto supplementation does not lead to prolong bleeding time compared to other fibrinolytic agents (Liu, Xing, Chang, Ma, & Liu, 2005). Therefore, it is safe to consume natto daily as a functional food (Liu, Xing, Chang, Ma, & Liu, 2005; Wang et al., 2009). Further, natto contains phytoestrogenic compounds genistein and daidzein which can act as a chemo protective agent. Also, natto contains ɣ-poly glutamic acid (ɣ-PGA) which is reported to help improve lipid metabolism and oxidation stress in over weight subjects (Taniguchi-Fukatsu et al., 2012). The composition and metabolites of the intestinal flora, was improved and the odour of faeces was reduced due to improved digestion by consuming natto (Akatsuka, 2000). Natto is a good source of lecithin, which may assist with reducing risk of arteriosclerosis and may help increase concentration ability. As natto contains high amount of vitamin K and K_2, it can assist in the prevention of calcification of soft tissue (blood vessels). Also, it has been found that vitamin K reduces heart attacks and cardiovascular diseases (Clouatre & Shastri, 2007). Further vitamin K supports

calcium resorption, increase bone mineral density and therefore important for overall bone health (Kaneki et al., 2001). Generally fermented foods contain vitamin K and natto has 870 µg 100 g^{-1} of vitamin K_2, which is higher than any other fermented food, such as cheese and miso (Minton, 2009). As natto (along with soy protein in general) has beneficial effects on heart health and blood pressure, the Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF) has recommended consuming natto daily (Ministry of Agriculture, Forestry and Fisheries, 2010).

2.6 Conclusion

This literature review summarizes the nutritional importance of legume lupin and the potential benefit of its fermented products lupin natto and tempeh. Fermentation is a valuable bio-process which enhances nutritional quality and functional food value of legumes and it improves their nutrient bioavailability and digestibility. In this chapter, the benefits of lupin as a nutritious legume due to its high content of protein, dietary fibre, important dietary minerals and phytochemicals has been evaluated. It is argued that lupin seed could be a good substitute for soybean and that high nutritional quality natto and tempeh analogues from lupin should be able to be prepared successfully.

CHAPTER 3

CHARACTERIZATION OF LUPIN NATTO AND TEMPEH AS AFFECTED BY LUPIN SOURCE, FERMENTATION TIME AND MICROBIAL STRAIN

3.0 Connecting statement

This chapter details the development and characterisation of the natto and tempeh analogues from lupin seed (whole seed and dehulled seed). Laboratory batches of lupin natto and lupin tempeh were produced. Samples were withdrawn from incubation oven at regular intervals and physical, chemical and organoleptic tests were performed. These experiments produced data required to characterise the so-called products novel lupin natto and lupin tempeh.

3.1 Introduction

Despite having minor compositional differences such as containing low sugars, fat and phytoestrogens lupin is a protein rich legume which is much similar to soybean (Sirtori et al., 2004; Sirtori, Arnoldi, & Johnson, 2005;Jayasena, Chih and Nasar-Abbas, 2010)). Also, lupin seeds are alike soybean in its size and appearance. Anti-nutritional factors which are common to most of the legumes are very low in lupin (Fudiyanasyah, 1995). Therefore, lupin will be an interesting alternative to soybean in the Asian fermented legume food market. Lupin production in Australia remains largely adequate to guarantee a huge export market over period of time. Therefore, which in turn must innovate to

produce attractive lupin based protein rich food ingredients and marketable food can be developed and proffer to consumers. In this regard, substitute lupin in Asian traditional soybean fermented foods such as natto and tempeh would be an interesting opportunity as there are many advantage of using lupin over soybean in this industry. For example; Despite having similar qualities to soybean, cost of lupin on the world market is usually 70-75% lower than that of soybean. Also, lupin *(L.angustifolius*) contains lower anti nutritional factors especially sweet lupin (*L.angustifolius*) varieties compared to soybean. For instance; lupin contains lower phytic acid which reduces the bioavailability of minerals, lower content of trypsin inhibitors and very low level of lectins which are gastric irritants. Petterson & Crosbie (1990) described that as lupin does not have a distinct beany flavour like soybean does and may have a greater acceptance in Western countries. Further lupin has similar nutritional and fermentation profiles to soybean (Sardjono et al., 1998). As described in chapter 2, tempeh is a traditional Indonesian fermented food which is produced from de-hulled soy bean seed fermented with the mould *R. oligosporus* (Bavia et al., 2012). Lupin tempeh was been prepared by Yu (1988) who reported that the mould grows faster on lupin than soya and the product had strong nutty flavour. Further the mould mycelia produced a firm white cake, the shelf-life of which was longer than that of the soy temphe (Yu, 1988). Comparable results were obtained by Hung et, al. (1990) follwing a commercial soy bean tempeh production methodology. Kidby et, al. (1977) reported that mouth feel, flavour, colour and appearance were better in lupin tempeh over soybean tempeh. However, a drawback of this study was that the all panellists were from Western countries with no previous knowledge of tempeh. Agosin

et, al. (1989) studied the chemical and nutritional characteristics of tempeh of *Lupinus. albus*. There were three main phases of fermentation, which include: first phase (first 17 h) which showed high mycelial growth, high proteolytic and lipolytic activity; second phase showed less mould activity; and the third phase exhibited increased lipolysis, free fatty acid and increased ammonia. Third phase indicated the loss of tempeh quality. Fudiyansyah (1993) produced lupin tempeh using commercial inocula. Panellists from various countries of birth preference the lupin tempeh over soybean tempeh. In another experiment, lupin tempeh was prepared in a soybean tempeh factory using similar techniques. However, there was no preferences shown for any tempeh. In contrast to the other studies by Hung et al.,(1990) and Yu ,(1988), lupin tempeh produced by Buoghton, Fairbrother and Petterson, (1994) was studied for shorter period of time making it difficult to compare.

Tempeh has become a major protein source in Indonesia due to its affordability compared to animal protein sources (Karyadi & Widjaja, 1996). Also, it is rich in some bioactive compounds such as peptides, phyoestrogens genistein and daidzein, vitamins i.e. B12, riboflavin and bioavailable minerals (Haron et al., 2009). Tempeh is prepared from cooked de-hulled soybean fermented with the mould *R. oligosporus.* Although it is a product of a mixed culture fermentation *R. oligosporus* is considered as the dominant tempeh forming mould (Babu, 2009). However, no previous lupin tempeh study has evaluated the presence of bioactive compounds, phytoestrogens, genistein and daidzein
Tempeh analogues from different substrate other than soybean have been investigated and listed as follows (Steinkraus, 1983).

Table 3.1 Tempeh analogues from different substrate other than soybean

Alternate substrate (name of tempeh)	Description
1. Tempeh kedele	is produced from soybean and consumed primarily by vegetarians in Indonesia, Malaysia the West Indies and several Western countries. Beans are boiled, drained, cooled and inoculated, wrapped in banana leaves or plastic containers and incubated for two days.
2. Tempeh mata kedele	Soybean hulls are fermented to prepare tempeh and similar procedures to above is followed.
3. Tempeh gembus	The solid refuse of tahoo (soybean curd) factories are used to produce tempeh gembus. The waste is pressed to expel to remove water, steamed cooled, inoculated, wrapped in banana leaves and incubated for two days.
4. Tempeh bongkrek	These is made from residue of coconut oil or milk extraction and above similar procedure are followed.

5. Oncom	ground peanuts are used to prepare this. Sometimes other raw materials such as tapioca, soybean curd may be included. Also, different inocula such as Neurospora can be used instead of Rhizopus.
6. Tempeh kecepir	The seeds of winged bean (Psophocarpus tetragonolobus) are used as raw material. The seeds are washed, boiled then the seed coat is removed, soaked and cut into smaller pieces before inoculation. It is then incubated for 48 h.
7. Wheat tempeh	Wheat is used as raw material and soaked, boiled and cooled wheat was inoculated with R. oligosporus. Product has popcorn flavour.
8. Tempeh from mung bean	Boiled mung bean is pressed to expel to remove water, steamed cooled, inoculated, wrapped in banana leaves and incubated for two d.
9. Yellow pea tempeh	The yellow pea is soaked, boiled, inoculated 40 h to produce this.
10. Tempeh from broad beans, cow peas, barley	Similarly, beans are soaked, boiled and inoculated for 40-48 h

Products such as wheat tempeh and mung bean tempeh have been prepared using laboratory techniques and they were deemed not applicable for mass production or the cottage industries (Steinkraus, 1983). Chick peas and horse beans have been used to prepare tempeh and the product had stronger flavour compared to soy tempeh (Robinson & Kao, 1977). Also, Shambuyi et al. (1992) used cassava, cowpea defatted peanuts and rice as raw materials for the production of tempeh. The mould growth was better in cassava and rice at 37 °C.

As described in chapter 2, natto is a traditional food from Japan which made from boiled whole soy bean fermented for about 24 h with *Bacillus subtilis/Bacillus natto* (Sumi, Hamada, Tsushima, Mihara, & Muraki, 1987b). Natto has characteristics flavour (cheese like) and a soft and sticky texture. Natto analogues from other legumes such as chick pea, red bean, pigeon pea, locust bean has been reported. Natto powder and nattokinase has been prepared from chickpea powder. The prepared product has been found to lower blood glucose on the basis of dissolving thrombus and the product did not have any ammonia odour (Sumi, Hamada, Tsushima, Mihara, & Muraki, 1987b). Furthermore, *B. subtilis* fermented red bean (50% ethanol extraction) had higher antioxidant activities (Chou, Hsu, Tseng, & Chung, 2010). In another study in Taiwan natto red bean was prepared and it was found that the purified fibrinolytic enzyme was a subtiline like serene protease (Chen et al., 2012). The natto pigeon pea with immobilized *Bacillus natto* were optimized and sensory evaluation indicated that the product was acceptable (Feng et al., 2015).

However, lupin has never been used in natto production although it has some suitable nutritional composition (high protein) for natto formation. Therefore, the aims of this study were as follows:

(i) to produce lupin tempeh from two lupin sources whole seed (WS) and dehulled seed (DHS) and two strains of moulds and characterise the final product quality using several parameters such as moisture, pH, firmness, ammonia content and microbial population (using total plate count) and some organoleptic measurements such as odour and level of mycelia growth.

(ii) to develop laboratory scale lupin natto from two sources whole seed (WS) and dehulled seed (DHS) and different bacterial strains (type of inoculum and level of inoculum) and investigate the effect of fermentation time on final product physical, organoleptic and chemical quality parameters.

3.2 Materials and methods

3.2.1 Materials

The ASL seeds (*L. angustifolius*) investigated in this study were obtained from a commercial dealer CBH group, Perth, Western Australia. Both whole lupin seed (lupin seed with the hull) and dehulled lupin seed (lupin seeds/ kernels without hull refers to the dehulled lupin) were separately purchased as whole seed and dehulled seed and test samples were prepared individually. The seeds were stored in airtight plastic containers at room temperature. Commercial soybean tempeh (Margret River tempeh, Wembley, Western Australia) and natto (Asaichban Kotsubu natto, Nippon food supplies, Osborne park, Western Australia) were purchased from an Asian market Perth Western Australia. USA commercial starter (USC) (Cultures for health, Morrisville, North Carolina) and another commercially available Indonesian commercial starter culture (ISC) (Ragi tempeh, Bandung, Indonesia) were used as *R. oligosporus* stater culture for making tempeh. Rose Bengal Chloramphenicol agar (RBC Agar) was purchased from, Sigma Aldrich, New South Wales, Australia. (6633^{TM}/10000 CFU vitroid form) was purchased from Sigma-Aldrich Australia and ATCC 15245 *B. subtilis natto* (also known as *Bacillus natto* Sawamura) was directly ordered from American Type Culture Collection USA used as inoculum for producing natto.

Figure 3.1 Lupin tempeh

Source: http://urbanlocavore.com.au/shop/margaret-river-tempeh/

3.2.2 Methods

3.2.2.1 Preparation of lupin seed as fermentation medium

After removing impurities lupin seeds were washed and soaked in water at room temperature (23 °C ±2 °C) overnight (16-18 h) Then the seeds were boiled at 100 ^{o}C for 60 min and pressure cooked (Tefal Secure 5 Neo, Groupe SEB Australia Pty Ltd, New South Wales, Australia) for 60 min at 121 °C (1.2 kg cm^2).

3.2.2.2 Preparation of *Bacillus subtilis* strains (inoculum)

Bacterial strains were cultured at 34 °C in nutrient broth (Sigma Aldrich, New South Wales, Australia), containing 5 g NaCl, 5 g peptone 2.5 g yeast extract) purchased in its dehydrated form. The dehydrated broth was dissolved in 1000 ml deionised water (dH_2O) and autoclaved (AC3D, Siltex pvt Ltd, Melbourne, Australlia) at 121 °C for 20 min. Vitroid disc (vitroid discs contain bacteria or fungi in a solid water soluble matrix. Microorganism in this form are stable for at least one year and are in a viable stage) (*Bacillus* strain ATCC^{R} 6633^{TM}) was added to the media when cooled to 35-40 °C and incubated at 34 °C in a shaking water bath (OLS200Grants Instruments Cambridge Ltd, Shepreth, England) at 150 rpm for 48 h. The method of Wei et al. (2001) was used to prepare inoculums. Similarly, ATCC 15245 starter culture grew in the nutrient broth and. The cells produced from 18h of incubation period which was highest viable population (6.4×10^8 CFU g^{-1}) were harvested. Viable population were determined using plate count method. Harvested samples (ferment) were refrigerated to suppress the further bacterial growth (to achieve similar cell count). Then the nutrient broth solution was centrifuged at 12000 rpm (20100 g) (Beckman J2, PALO, ALTO, California, USA) for 25 min. The cell pellet were aseptically collected and diluted in 20 mL sterile phosphate buffer solution (0.85% Nacl and 0.1% peptone mixture) to prepare as suspension, which was used for inoculating the boiled and steamed lupin (Wei et al., 2001).

3.2.2.3. Preparation of *R. oligosporus* inoculum

USA commercial starter (USC) (*R. oligosporus)* starter culture was purchased from Cultures for Health, Morrisville NC, USA. Starter was in freeze dried powder form and required amount (e.g 0.2 mg 100 g^{-1}) was accurately weighed and used when inoculating the samples. Indonesian commercial starter (ISC) of *R. oligosporus* was in powder form and was weighed required milligrams for the experiment.

3.2.2.4 Preliminary studies

Preliminary studies were to find out suitable microbial strain, inoculum level, and precooking method to prepare laboratory scale lupin tempeh and lupin natto using both whole seed and dehulled seed. Indonesian starter culture (ISC) lupin tempeh, 0.2 g 100 g^{-1} inoculum level and pressure cooking method were selected as the suitable conditions (data not presented). Similarly, it was found that bacteria strain ATCC15245, inoculum level 10^{4-6} CFU g^{-1} and pressure cooking (Tefal Secure 5 Neo, Groupe SEB Australia Pty Ltd, New South Wales, Australia) method produced better quality lupin natto (data not presented).

To make sure excellent quality fermented product is obtained, dirt and broken seed pieces were removed by aspiration and screening lupin seeds. Lupin seeds (whole seed and dehulled processed separately) 500 g were weighed in a 2000 mL beaker and soaked in an excess (volume of 3 times of the bean weights) of water for 18 h. White vinegar (30-40 mL of commercial / Black and Gold brand) (Acetic acid, pH 4) was added to (500 g lupin) make acidic medium and promote acidic fermentation (Deshpande, 2000). The pH of the soaking medium was monitored placing a calibrated pH metre probe into the beaker. After subsequent washing, seeds were cooked (100 °C) for 1 h, drained and cooled to 37 °C. There were three separate experiments (to compare the different stain, inoculum level and seed processing method) were performed to confirm preliminary data and to determine the suitable fermentation parameters to prepare lupin tempeh. In the first experiment, cooled seeds were inoculated with USA starter culture(USC) (*R. oligosporus)* starter culture and Indonesian starter culture (ISC) of *R. oligosporus* separately (according to our preliminary study data, inoculum level, 0.2 g 100 g^{-1} and 30 min pressure cooking method were used as other experiment conditions). There were three inoculum levels (0.1 g 100 g^{-1}, 0.2 g 100 g-1 and 0.3 g 100 g^{-1}) were employed in second experiment (according to our preliminary study data ISC and 60 min pre-cooking method were used as other fermentation parameters). In third experiment, two pre-cooking methods; 60 min boiling and 30 min pressure cooking (Tefal Secure 5 Neo, Groupe SEB Australia Pty Ltd, New South Wales, Australia) were performed and other fermentation parameters were ISC and 0.2 g 100 g^{-1} inoculum level respectively. Then inoculated seeds were placed in perforated clear plastic bags (Ortega-david & Rodríguez-stouvenel, 2013). Packages were incubated at 37 °C in humidity (85%) controlled

incubator (D-91126 Schwabach FRG, Germany) and fermented lupin samples were aseptically collected from 0 to 90 h at six h intervals. Both whole seed and dehulled dee samples were freeze dried (-30 °C, vacuum: 0.37 mbar) ground using a mill (1090 Cemotec, Foss Tecator AB, Hoganas, Sweden) and stored in the freezer (-20 °C) for future analysis (Deshpande, 2000)

Figure 3.2 Dehulled lupin tempeh 0 h fermented

Figure 3.3 Whole seed lupin tempeh 0 h fermented

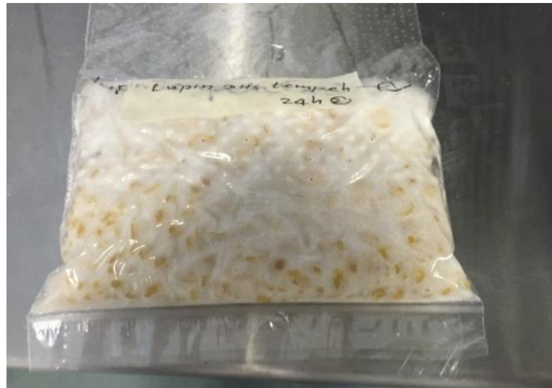


Figure 3.4 Dehulled lupin seed ferment with *R. oligosporus* at 24 h with white mould mycelia

Figure 3.5 whole seed lupin seed ferment with *R. oligosporus* at 24 h with white mould mycelia

3.2.2.5 Preparation of lupin natto

After removing impurities,whole lupin seeds were washed and soaked in water at room temperature (23 °C-25 °C) overnight. Then the seeds were boiled for 1 h and air dried for 20 min until temperature of the beans reach 30 °C-40 °C. 5 mL of above inoculum preparation was added to 120 g of steamed/boiled beans.

Inoculated seeds were placed in perforated plastic bags and incubated at 37 °C in humidity control oven under 85-90% RH. Samples were collected at 6 h intervals 0– 60 h . All samples were prepared in triplicate and prior to analysis, they were freeze dried and then milled into fine particles using laboratory grinder (1090 Cemotec, Foss Tecator AB, Hoganas, Sweden) (100% through 100-micron sieve). Then smaple were stored in the -20 °C freezer.

Similar procedure was follwed for dehulled seed as well.

As explained in above, three experiments were conducted with two strains (ATCC 15245 and ATCC 6633), three inoculum levels (10^4, 10^6,10^{8} CFU g^{-1}) and two pre-cooking methods (60 min boiling and 30 min pressure cooking). Bacterial strains were cultured at 34 °C in nutrient broth (Sigma Aldrich, New South Wales, Australia), containing 5 g NaCl, 5 g peptone 2.5 g yeast extract) purchased in its dehydrated form. The dehydrated broth was dissolved in 1000 mL deionised water (dH_2O) and autoclaved (AC3D, Siltex pvt Ltd, Melbourne, Australia) at 121 °C for 20 min. Vitroid disc (vitroid discs contain bacteria or fungi in a solid water-soluble matrix. Microorganism in this form are stable for at least one year and are in a viable stage) (Bacillus strain ATCCR 6633TM) was added to the media when it cooled down to 35-40 °C and incubated at 34 °C in a shaking water bath (OLS200Grants Instruments Cambridge Ltd, Shepreth, England) at150 rpm for 48 h. The method of (Wei et al., 2001) was used to prepare inoculums. Similarly, ATCC 15245 starter culture grew in the nutrient broth and. The cells produced from 18 h of incubation period which was highest viable population (6.4 x 10^{8} CFU g^{-1}) were harvested. Viable population were determined using plate count method. Harvested samples (ferment) were refrigerated to suppress the further bacterial growth (to achieve similar cell count). Then the nutrient broth solution was centrifuged at 12000 rpm (20100 g) (Beckman J2, PALO, ALTO, California, USA) for 25 min. Cell pellet were aseptically collected and diluted in 20ml sterile phosphate buffer solution (0.85% NaCl and 0.1% peptone mixture) to prepare as suspension, which was used for inoculating the boiled and steamed lupin (Wei et al., 2001).

Figure 3.6 Boiled dehulled lupin before fermenting

3.2.2.6 Preparation of lupin tempeh

To make sure excellent-quality, fermented product is obtained, dirt and broken seed pieces were removed by aspiration and screening. Lupin seeds (whole seed and dehulled processed separately) 500 g were weighed in a 2000 mL beaker and soaked in an excess (volume of 3 times of the bean weights) of water for 18 h. White vinegar (30-40 mL of commercial / Black and Gold brand)(Acetic acid, pH 4) was added to (500 g lupin) make acidic medium and promote acidic fermentation (Deshpande, 2000). The pH of the soaking medium was monitored placing calibrated pH metre probe into the beaker. After subsequent washing, seeds were cooked (100 °C) for 1 h, drained and cooled to 37 °C. There were three separate experiments (to compare the different strain, inoculum level and seed processing method) were performed to confirm preliminary data and to determine the suitable fermentation parameters to prepare lupin tempeh. In first experiment, cooled seeds were inoculated with USC (*R. oligosporus)* starter culture and ISC of *R. oligosporus* separately (according to our preliminary study data, inoculum level,

0.2 g 100 g^{-1} and 30 min pressure cooking method were used as other experiment conditions). There were three inoculum levels (0.1 g 100 g^{-1}, 0.2 g 100 g^{-1} and 0.3 g 100 g^{-1}) were employed in second experiment (according to our preliminary study data ISC and 60 min pre-cooking method were used as other fermentation parameters). In third experiment, two pre-cooking methods (60 min boiling and 30 min pressure cooking) were performed and other fermentation parameters were ISC and 0.2 g 100 g^{-1} inoculum level respectively. Then inoculated seeds were placed in perforated clear plastic bags (Ortega-david & Rodríguez-stouvenel, 2013). Packages were incubated at 37 °C in humidity (85%) control incubator (D-91126 Schwabach FRG, Germany) and fermented lupin samples were aseptically collected from 0 to 90 h at six hours intervals. Samples were freeze dried (-30 °C, vacuum: 0.37 mbar) ground (1090 Cemotec, Foss Tecator AB, Hoganas, Sweden) and stored in the freezer (-20 °C) for future analysis (Deshpande, 2000).

3.2.2.7 Determination of total plate count

Viable bacteria populations of the inoculum and the final products were determined by the aerobic plate count procedure using the spread plate method (Wei et al., 2001). Lupin natto final product (10 g) was weighed and aseptically blended in 90 mL of 0.1 peptone to make the first dilution. Serial dilutions were prepared using 1mL of sample and 9 mL of 0.1% peptone. The prepared dilution plated out up to 102-109 (Grubb, 1995). The samples were pipetted on to the agar plate and spread using plastic disposable sterile spreader. Plate count agar with dicloran was used to inhibit the mould growth. In each experiment, open and close controls were performed, and open control helped determining purity of the working surrounding and closed one was used to check the medium purity.

3.2.2.8 Determination of total mould count

Similarly, 10 g of lupin tempeh (48 h) was weighed and aseptically blended in 90 mL 0.1 % peptone to make the first dilution. Serial dilutions were made using 1 mL of tempeh sample and 9 mL of 0.1 peptone. Then 0.1 mL of the sample was pipetted on to surface of agar plate (RBC Agar, Rose Bengal Chloramphenicol Agar) and spreader using disposable plastic spreader. Each dilution on each of the medial was plated in triplicate. As indicated earlier, open and close controls were maintained to monitor working environment cleanliness and medium purity.

3.2.2.9 Inoculation and incubation and storing

When temperature of the beans reaches 30 °C-40 °C 5 mL of above inoculum preparation was added to 120 g of steamed/boiled beans (Deshpande, 2000; Wei et al., 2001). Inoculated seeds were placed in perforated plastic bags and incubated at 37 °C under RH in humidity control incubator (85-90%) (D-91126 Schwabach FRG, Germany). Samples were aseptically collected at 0 h (unfermented), 6 h, 12 h, 24 h, 30 h, 36 h, 42 h, 48 h, 54 h, and 60 h for analysis. The collected samples were freeze dried (-30 °C, vacuum: 0.37 mbar) ground and stored in the -20 °C freezer until required.

3.2.2.10 Determination of proximate composition and physico-chemical analyses

Proximate composition was determined using AOAC methods (AOAC, 2008). Moisture content was determined using oven (D-91126 Schwabach FRG, Germany) drying method and ash was determined by dry –ashing at 550 °C by method 923.03 (AOAC, 2008). Protein was measured (Kjeldahl digestion and distillation method; Nx5.44) according to AOAC method 920.87 (AOAC, 2008). Crude fat was determined by petroleum ether extraction (Buchi E-816, Flawil, Switzerland) using AOAC Method 945.16 (AOAC 945.16). Temperature changes were monitored using Humidity and Temperature Recorder (Log tag: Haxo-8).

3.2.2.11 Visual Analogue Scale (VAS) questionnaire for determining the growth and odour characteristics of lupin tempeh

Organoleptic changes i.e. odour and mycelium growth were performed by a semi-trained panellist. The panellists were trained as per AS 2542 for tempeh quality (aroma and flavour) determination, using soya tempeh as the reference. During this physical and organoleptic evaluation test, commercial soybean tempeh and natto were used as a reference material and three panellists were selected from the staff and students from the School of Public Health, Curtin University, Perth, Western Australia based on their knowledge of Tempeh. People with food allergy were excluded. During the VAS training, product characteristics and details of reference sample were showed and described in detail. They were taught how to answer to the VAS questionnaire appropriately. Three model trails were conducted under real evaluation conditions to familiarize the panellists. Trial results were disregarded.

In selecting which tempeh samples to be used in further analysing for selecting the best fermentation time for making tempeh (for lupin tempeh characterisation), the inoculated samples were collected at 6 h time intervals starting from 0-90 h during our pilot organoleptic experiment. Then the samples were tested by at least one experienced tempeh consumer in terms of appearance (mycelium growth) and aroma (odour). In the evaluation, the samples were presented to three evaluators which were asked to score the acceptability of samples in terms of above same VAS parameters using a 0 through 10 scale with 0 being off and 10 was given perfect with reference to commercial soybean tempeh from an Asian market Perth Western Australia. Mycelium growth and odour were monitored in three experiments such as different starter cultures, different inoculum levels and different cooking methods (60 min boiling and 30 min pressure cooking), to determine the characteristics of lupin tempeh.

3.2.2.12 Visual Analogue Scale (VAS) questionnaire for determining the growth and odour characteristics of lupin natto

Stickiness and odour (fresh /ammonical) were monitored in three independent experiment conditions such as different starter cultures, different inoculum levels and different cooking methods to determine the characteristics of lupin natto. As shown in Table.3.1 in the first experiment appearance/stickiness of lupin natto were recorded by the evaluator of VAS score. Whole seed lupin natto (WSLN) and dehulled lupin natto (DHLN) were prepared using starter culture ATCC 15245 and ATCC 6633 strain. Based on our preliminary experiment outcome, 10^{4-6} CFU g^{-1} inoculum level and pressure-cooked lupin beans were used at 37 °C incubation temperature.

Similarly, lupin natto samples were tested by at least one experienced natto consumer in terms of stickiness (drawing situation) and odour (fresh aroma or ammonical). In house, Visual Analogue Scale (VAS) questionnaire was used score the acceptability of samples in terms of odour characteristics (see appendix VAS form). There were three evaluators and they were asked to score the acceptability of samples in terms of VAS parameters using a 0 through 10 scale with 10 being off and 0 was given perfect with reference to commercial soybean natto from an Asian market Perth Western Australia. Stickiness and odour were monitored in three independent experiment conditions such as different starter cultures, different inoculum levels and different cooking methods, to determine the characteristics of lupin natto.

Reference sample

Commercial soybean tempeh was purchased from an Asian market Perth Western Australia.

3.2.2.13 pH measurements

Natto and tempeh samples (3 g) were mixed with 10 mL deionised water and homogenized using electrical homogenizer (PRO25D, Thomas Scientific, NJ, U.S.A). Then pH was measured using calibrated digital pH meter (Wei et al., 2001).

3.2.2.14 Firmness/hardness of cooked lupin, lupin natto and lupin tempeh

Instrumental textural properties of hardness were determined with TA-XT2i texture analyser (Stable Microsystems Ltd, Surrey, UK) with 5 kg load cell. Texture profile was analysed using 36 mm cylindrical aluminium probe. Firmness (g) was the peak force after first compression. Standard size cube (1 cm) samples were subjected to complete penetration of the probe. However, information on the detection limitations, accuracy and sensitivity were not available. Boiled and pressure-cooked lupin seeds were cooled down to room temperature before testing the firmness. The prepared products (lupin natto & tempeh) were stored in a refrigerator to inhibit the fermentation and were equilibrated to room temperature before testing the firmness by texture analyser (TA-XT2i Surrey GU7 1YL, UK). The results were expressed as the force (g) required pressing down sample.

3.2.2.15 Total Volatile Bases (TVB)

Lupin tempeh and natto samples (0 h, 12 h, 24 h, 36 h, 48 h) was accurately weighed (10 g) into a Kjeldahl distillation tube. Then 50 mL deionised water and 2 mg magnesium oxide (MgO) was added and mixed well. The distillation tube was attached to a steam distillation unit (Tecator Digestion System and a Kjeltec 1030 auto analyser). A flask with 25 mL boric acid and indicator (bromocresol green 0.1% and methyl red in ethanol) was placed at the condensing chamber. After opening steam, distillation was continued until 125 mL of distillate was collected. Then distillate was titrated with a standard solution of 0.01M HCl to red green end point (Fenner, 1965; Botta, Lauder, & Jewer, 1984; Castro et al., 2012).

3.3 Statistical Analysis

Data are presented as mean values with standard deviation. While t-Test was used to analyse Visual Analogue Scale, repeated measures ANOVA with within samples factors (for the time effect), between sample factors (for sources; WS and DHS) and their interaction (time x source) was used analyse chemical and physical tests differences. When main effects or interaction were significant, individual means were compared by Bonferroni test. ($P < 0.05$) was considered as significant. IBM SPSS statistics V.24 (IBM Corp. NY, USA) was used for all analyses.

3.4 Results and discussion

3.4.1 Visual Analogue Scale (VAS) questionnaire for determining the growth and odour characteristics of lupin Tempeh

As shown in Figures 3.7, 3.8 and 3.9, there were three separate experiments conducted to prepare whole seed lupin tempeh (WSLT) and dehulled seed lupin tempeh (DHLT) using two different starter cultures Indonesian I starter culture (ISC) and USA starter culture (USC), three inoculum levels and two seed pre-cooking methods. In the first experiment, lupin natto and tempeh were prepared using two inoculums.

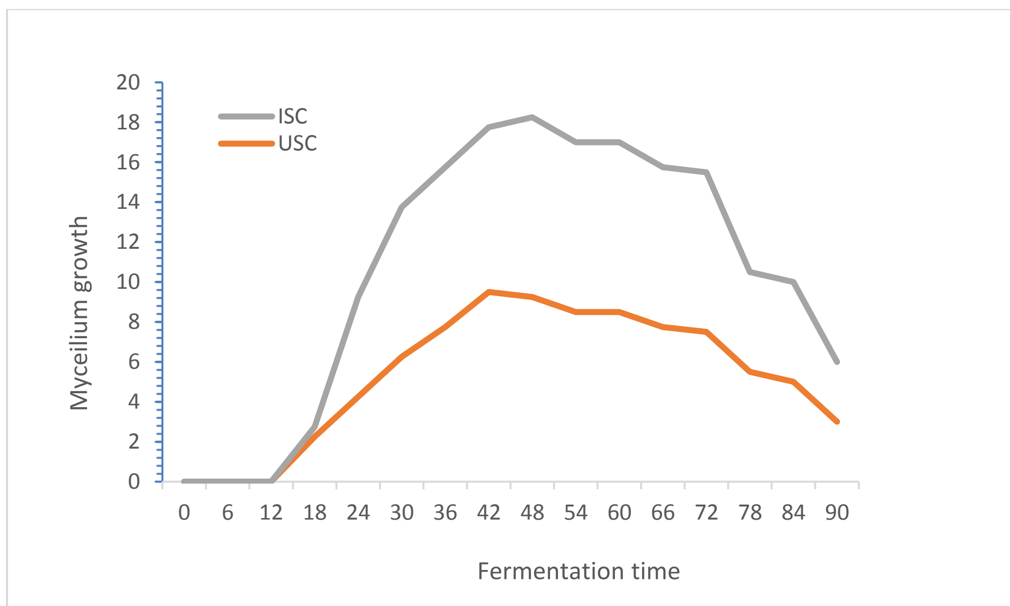


Figure 3.7 Mycelium growth on lupin tempeh[1] as scored by evaluators using in Visual Analogue Scale (VAS) questionnaire over the fermentation period; effect of inoculum type

inoculum level=0.2 mg 100 g^{-1}, pressure cooked lupin ISC, Indonesian I starter culture; USC, USA starter culture; WS, Whole seed; DHS, De-hulled seed;

[2] Means ± standard deviation(n=3)

Score,0= no growth, 10= thick mycelium growth covering the substrate

(ISC and USC) and other experimental conditions inoculum level 0.2 % and 30 min pressure cooking processing method were kept as constant at 34 °C, for both treatments according to our preliminary experiment outcome and following references (Coorey,1995; Embaby, 2010). In WSLT and DHLT prepared using Indonesian starter, white mycelium appeared at 18 h and 24 h and best mould growth was at 48 h and 42 h, respectively. Both WSLT and DHLT from USC produced white mycelium at 18 h and peaked at 48 h then reduced. Therefore, there was no noticeable difference of time which mycelium growth started between whole seed and de-hulled seed of USC. Also mould growth intensity (the amount of mycelium) of ISC lupin tempeh was similar to level of reference sample (commercial soy tempeh) but USC lupin tempeh did not show much mycelium growth compared to reference sample (Figure 3. 7). Tempeh prepared from ISC showed significantly higher ($P < 0.05$) mycelium growth compared to tempeh prepared from USC. Therefore, as shown in the graph ISC is better than USC with respect to mycelia growth on the substrate. In the second treatment, WSLT and DHLT prepared using three different inoculum levels (0.1, 0.2 and 0.3 mg 100 g^{-1}) and ISC and pressure cooking were kept constant for three treatments as other experimental conditions. As presented in Figure 3.8 when inoculum level 0.1 mg 100 g^{-1} were used in WSLT mycelium growth was started at 24 h and in DHLT it was delayed until 36 h. Maximum mycelium growth was reported at 36 h for WSLT and DHLT prepared from 0.1 mg 100 g^{-1} were not comparable to the reference soy tempeh (sensory score was 7.5 and 6, respectively).

The results of WSLT and DHLT prepared from 0.2 mg 100 g^{-1} showed that, WSLT and DHLT mycelium growth started at 18 h, 24 h, respectively. Both WSLT and DHLT showed maximum growth at 48 h. The amount of mycelium growth of both WSLT and DHLT were similar to the reference sample. Then, WSLT and DHLT were prepared from 0.3 mg 100 g^{-1} inoculum level and mycelium growth started 12 h in both WSLT and DHLT which is an early erupt of mycelium before the complete maturation of tempeh. Significantly higher (P<0.05) mycelium growth was observed between 42-48 h in both WSLT and DHLT. The amount of growth was similar to the reference soy sample. Considering the starting time for mycelium growth, maximum amount of mycelium and time, 0.2 mg 100 g^{-1} selected as the optimum level of inoculum for lupin natto production.

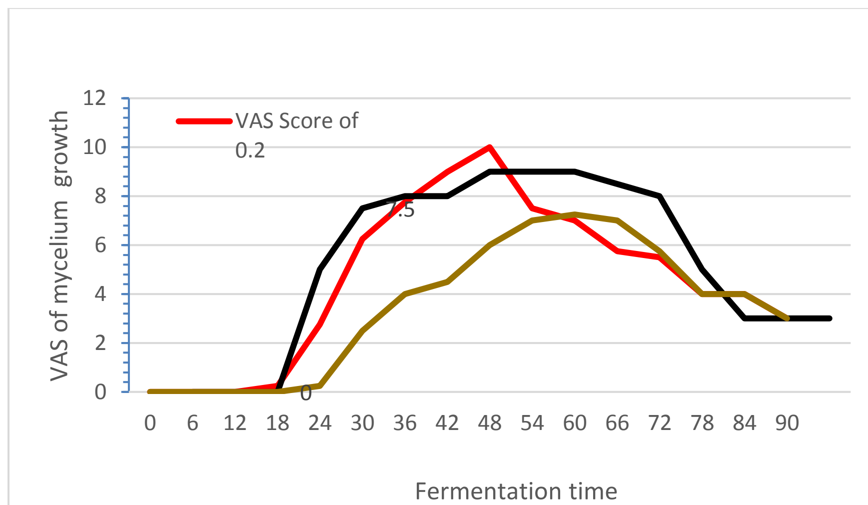


Figure 3.8 Mycelium growth on lupin tempeh[1] as scored by evaluators using in house Visual Analogue Scale (VAS) questionnaire over the fermentation period; effect of inoculum level.

[1] (ISC, Indonesian commercial stater, 34 °C, Pressure cooked lupin); WS, Whole seed, DHS, De-hulled seed; at 37 °C,

Score,0= no growth, 10= thick mycelium growth covering the substrate, +=0.5

Then in the third experiment two seed pre-cooking (60 min boiling and 30 min pressure cooking at 121 °C & 15 psi) methods were tested and as other experimental conditions, starter culture(USC) and inoculum level (0.2 mg 100 g^{-1}) were kept constant for both experiments (Figure 3.9). In boiled samples mycelium growth started at 24 h in WSLT and DHLT and pressure-cooked samples mycelium growth started at 18 h for both WSLT and DHLT. Also, mycelium growth was maximum at 24 h in both tests. However, the amount of mycelium growth of samples prepared from pressure cooking was similar to reference soy sample. Lupin tempeh prepared from boiling did not have enough growth compared to soy reference. Therefore, considering the starting time for mycelium growth maximum amount and time for mycelium growth, pressure cooking was selected as suitable pre-seed cooking method for preparing lupin tempeh.

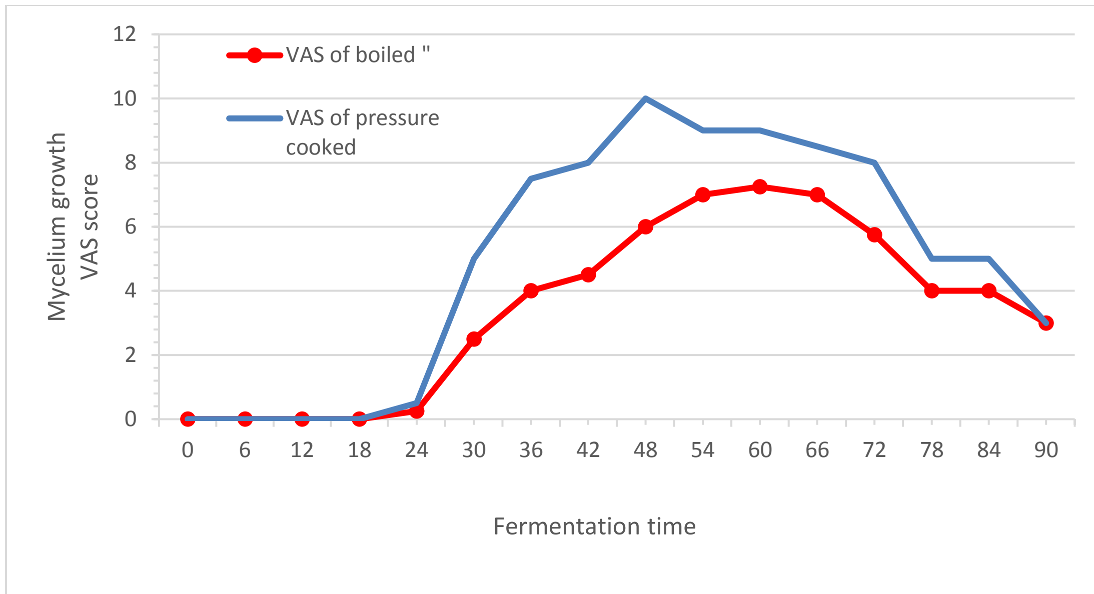


Figure 3.9 Mycelium growth on lupin tempeh1 as scored by the evaluators using in house Visual Analogue Scale (VAS) questionnaire over the fermentation period; effect of seed pre-cooking method

[1] ISC, Indonesian commercial stater, 34 °C, Pressure cooked lupin; WS, Whole seed, DHS, De-hulled seed; at 37 °C,
Score,0= no growth, 10= thick mycelium growth covering the substrate.

Similarly, as shown in Tables 3.2, 3.3 and 3.4 sensory evaluation of ammonical odour was performed by three evaluators who were asked to score the acceptability of samples in terms of ammonical odour using a 0 through 10 scale with 0 being perfect and 10 is off. Ammonical odour was first detected at 54 h in WSLT and DHLT from Indonesian stater while it was 42 h and 48 h for WSLT and DHLT from USA starter (Table 3.2). This reflects that lupin tempeh from Indonesian starter produces better quality lupin tempeh than USA starter as the appearance of ammonical odour leads to consumer unacceptability (Gunawan-Puteri, Hassanein, Prabawati, Wijaya, & Mutukumira, 2015).

In the second experiment, the effect of amount of inoculum lover the fermentation time on developing lupin tempeh was studied and pressure cooking processing method and Indonesian starter culture were used according to our preliminary experiment outcome. Three inoculums levels 0.1, 0.2 and 0.3% were tested and at 0.1% mould growth was slow and growth started at 24h in WSLT and in DHLT it was delayed until 36 h (Table 3.3). It was peaked at 48 h, but mould growth intensity was lower than 0.2 and 0.3%. The inoculum level 0.2% and 0.3% showed maximum mycelium growth at 48 h and 42 h respectively and mycelium growth intensity was similar to reference sample. However, in inoculum level 0.3% mycelium growth started as early as 12 h and because of that there is lesser time for maturation of lupin tempeh.

Similarly, the effect of level of inoculum on the odour of WSLT and DHLT over the fermentation time was studied. Least ammonical odour was noticed in 0.1% in which its first ammonical odour was detected at 66 and 60 in WSLT and DHLT respectively. In the sample with 0.2% inoculum level first appearance of ammonical odour happened at 54 h in DHLT and 48h in WSLT. Again, the sample with 0.3% inoculum level it was early as 42 h in both WSLT and DHLT which means early deterioration of the product. As the level of inoculum increase rate (mould growth is higher in 0.3 compared 0.1 and 0.2) of growth of mould is high initially. Therefore, WHLT and DHLT prepared from 0.3 mg 100 g^{-1} has rapid mould growth. As a result of rapid growth, these samples have produced ammonical odour at 42 h which is a sign of early spoilage. Hence, these samples are beyond the acceptable level for the consumption. Thirdly, the effect of processing methods (30 min pressure cooking and 60 min boiling) on mycelium growth of WSLT and DHLT over the fermentation time was determined and inoculum level of 0.2% and ISC were used according to our preliminary experiment outcome (Table 3.4). Appearing of white mycelium started at 24 h in both WSLT and DHLT in boiled samples. The appearance of mycelium in the pressure-cooked samples occurred earlier than boiled ones (18 h). However, mould-growth intensity of both WSLT and DHLT samples prepared from 30 min pressure- cooking was almost similar to reference sample whereas samples prepared from 60 min boiling did not show much mycelium growth compared to reference sample (commercial soybean tempeh). Therefore, from the mycelium growth perspective 30 min pressure-cooking produced better quality lupin tempeh. Hence, better consumer acceptability can be expected from pressure- cooking. The effect of cooking method

on odour of WSLT and DHLT were monitored over the fermentation time. Samples prepared from 60 min boiling showed better results than 30 min pressure cooking as it took 54 h to express ammonical odour while WSLT and DHLT from 30 min pressure cooking expressed unacceptability at 42 h and 48 h respectively.

According to above VAS outcome lupin tempeh samples which were prepared from lupin seed pressure cooked for 30 min, fermented up to 48 h at 37 °C was selected as the optimum conditions for preparing lupin tempeh. Other physical, chemical and biochemical reactions were performed based on this selection.

Table 3.2 Ammonical odour of lupin tempeh[1] as detected and scored by evaluators using Visual Analogue Scale (VAS) questionnaire over the fermentation period.; effect of inoculum type

Fermentation time (h)	VAS score of ammonical odour (Inoculum type)			
	ISC		USC	
	WS	DHS		WS
0	0	0	0	0
6	0	0	0	0
12	0	0	0	0
18	0	0	0	0
24	0	0	0	0
30	0	0	0	0
36	0	0	0	0
42	0	0	0	0.5
48	0	0	0.5	0.5
54	0.5	0.5	1	1
60	1	2	3	3
66	3	5	5	6
72	9	8	7	6
78	10	10	10	10
84	10	10	10	10
90	10	10	10	10

ISC Indonesian commercial Starter; USC, USA commercial starter; WS, Whole seed; DHS, De-hulled seed Commercial Starter;

Score,0= no growth, 10= thick mycelium growth

[1] at 34 °C, 0.2 mg 100 g^{-1}, pressure cooked lupin seeds.

Table 3.3 Ammonical odour of lupin tempeh1 as detected and scored by evaluators using Analogue Scale (VAS) questionnaire over the fermentation period.; effect of inoculum levels

Fermentation time (h)	Inoculum level(mg 100 g^{-1})					
	0.1		0.2		0.3	
	WS	DHS	WS	DHS	WS	DHS
0	0	0	0	0	0	0
6	0	0	0	0	0	0
12	0	0	0	0	0	0
18	0	0	0	0	0	0
24	0	0	0	0	0	0
30	0	0	0	0	0	0
36	0	0	0	0	0	0
42	0	0	0	0	0.5	0.5
48	0	0	0.5	0	1	1
54	0	0	1	0.5	3	3
60	0	0.5	3	1	6	6
66	0.5	1	6	3	9	9
72	1	3	6	6	10	10
84		5	10	10	10	10
90	6	6	10	10	10	10

WS, Whole seed, DHS, De-hulled seed; Score,0= no growth, 10= thick mycelium growth
VAS, Visual Analogue Scale
[1] 34 °C, ISC, pressure cooked lupin seeds.

Table 3.4 Ammonical odour of lupin tempeh as detected and scored by evaluators using VAS questionnaire over the fermentation period.; effect of seed pre-cooking method(processing)

Fermentation time (h)	odour Processing method/cooking			
	Boiling		Pressure cooking	
	WS	DHS	WS	DHS
0	0	0	0	0
6	0	0	0	0
12	0	0	0	0
18	0	0	0	0
24	0	0	0	0
30	0	0	0	0
36	0	0	0	0
42	0	0	0	0.5
48	0	0	0.5	0.5
54	0.5	0.5	1	1
60	1	2	3	3
66	3	5	5	6
72	9	8	7	6
78	10	10	10	10
84	10	10	10	10
90	10	10	10	10

[1] at 34 °C, 0.2 mg 100 g^{-1}, ISC; WS, Whole seed, DHS, De-hulled seed Commercial Starter
Score,0= no growth, 10= thick mycelium growth
VAS, Visual Analogue Scale

3.4.2 Development of stickiness and odour characteristics of lupin natto as determined by Visual Analogue Scale (VAS)

High quality natto , should be covered with a white coloured mucous substance. Also, it has a characteristic flavour, palatably soft texture, light yellow colour and able to generate sticky mass when stirred. Experimental samples were compared with original soy natto and both WSLN and DHLN prepared from ATCC15245, showed stickiness at 12 h and improved the quality until 18 h. Then gradually decrease with the time. The action of ATCC 6633 strain was slow compared to ATCC 15245 and even after 6 h of fermentation. The stickiness was low in both WSLN and DHLN (in ATCC 6633) (Table 3.5). Best sample was found at 24 h and still the quality of the sample was not to the standard of reference sample (commercial soya natto). In the second experiment, pressure cooked lupin beans fermented with different inoculums level (10^{2-4}, 10^{4-6}, 10^{6-8} CFU g^{-1}) of ATCC 15245 *Bacillus subtilis* (natto) strain at 37 °C and samples were drawn from 0-60 h (Table 3.4). The samples prepared from inoculum level 10^{2-4} CFU g^{-1} took longer time to show stickiness. The best lupin natto sample was recorded at 30 h. However, the quality of the product (max value 7.5) was low compared to reference sample (VAS score 10). DHNL also showed similar results and stickiness started 12 h with very low quality and maximum was at 24-30 h (6.0) which is lower than the reference sample (VAS score 10). The lupin natto prepared from 10^{4-6} CFU g^{-1} ATCC 15245 showed stickiness less than 6 h and at 18 h reached to peak value. The natto samples prepared from inoculum 10^{6-8} CFU g^{-1} showed early spoilage appearance and compared to reference sample it did not show proper stickiness and appearance of natto. Therefore 10^{4-6} CFU g^{-1} can be selected as

the best inoculums level for production of natto from lupin in terms of appearance or the stickiness. In the third experiment two types of processing (60 min boiling and 60 min pressure cooking) were used (Table 3.5). Here inoculum level 10^{4-6} CFU g^{-1} of strain ATCC 15245 was used and medium temperature was 37 °C. Lupin natto prepared from pressure cooked beans showed highest stickiness at 18 h and it was similar to standard reference sample. As shown in Table 3.5 both WSLN and DHLN from pressure cooked lupin bean were better than boiled lupin beans samples. Therefore, results indicated that pressure coking is the preferable method for preparing natto.

As shown in Table 3.6 lupin natto was prepared from different experiment conditions as explained in previous experiment. In this experiment, quality of lupin natto was evaluated in terms of presence of ammonical odour. Lupin natto prepared from inoculum level 10^{4-6} CFU g^{-1} of ATCC 15245 at 37 °C from pressure cooked beans showed better quality over ATCC 6633 as ATCC 15245 did not have even slight ammonical odour until 18 h for DHLN and until 12 h for WSLN. In the second experiment lupin natto was prepared with three different inoculums levels (10^{2-4}, 10^{4-6}, 10^{6-8} CFU g^{-1}) of ATCC 15245 and as the seed pre-cooking method pressure cooking was used. Medium temperature kept constant at 37 °C. Samples were drawn from 0-60 h. Lupin natto prepared from inoculum level 10^{6-8} CFU g^{-1} showed bad quality as ammonical odour started as early as less than 6 h for both WS and DHS. There was no difference between 10^{2-4} and 10^{4-6} CFU g^{-1} on time producing ammonical odour. This shows that the suitable or better quality lupin natto can be produced from inoculums level 10^{4-6} CFU g^{-1}. Therefore, optimum inoculum level was 10^{4-6} CFU g^{-1}.

Lupin natto samples prepared using pressure cooked and boiled lupin seed separately and in both experiments ATCC 15245 (10^{4-6} CFU g^{-1}) was used at 37 °C. Fermented lupin natto samples were withdrawn six hourly from 0-60 h. Results indicated that (Table 3.6) pressure cooking method produced better quality natto as DHLN produced ammonical odour only at 24 h whereas boiled samples started giving off ammonical odour as early as 12 h. Therefore, best processing method for preparing lupin natto with excellent quality is, pressure cooking method and inoculums level 10^{4-6} CFU g^{-1}. Therefore, in terms of formation of stickiness and aroma pressure cooked lupin beans, ATCC 15245 strain and 10^{4-6} CFU g^{-1} inoculum levels were the best conditions for preparing best quality natto compared to reference commercial soy natto.

Table 3.5 Appearance (stickiness) of the lupin natto ferment over the fermentation time as scored by the evaluators using VAS, Visual analogue scale[2]

Fermentation (h)	Inoculum type				Inoculums level (CFU g^{-1}) ‡						Processing method/cooking †			
	ATCC 15245		ATCC 6633		10^{2-4}		10^{4-6}		10^{6-8}		Boiling		Pressure cooking	
	WS	DHS	WS	DHS	WS	DHS	WS	DHS	WS	DHS	WS	DHS	WS	DHS
0	0	0	0	0	0	0	0.5	1	1	2	0	0	1	1
6	2	4	1	3	0	0	3	4	5	7	1	2	3	4
12	4	10	3	6.5	2	3	5	9	9	9	3	5	4	5
18	10	9	6	8	3	5	9	10	9	9	5	8	8	10
24	9	9	5	9	5	6	5	9	8	8	8	8	9	8
30	9	8	7.5	8	7.5	6	7.5	8	8	8	8	7	8	7
36	9	7.5	8	8	5	4	8	7.5	7	7	7.5	7	7.5	7
42	7	6	8	7	5	4	7	7	6	6	8	6	7	5
48	6	5	9	7	4	3	5	5	5	5	6	4	5	4
54	6	5	7	5	3	3	3	3	3	3	5	3	3	2
60	4	3	5	3	3	3	3	3	2	2	5	3	3	2

ISC, Indonesian commercial Starter; USC, USA commercial stater; WS, Whole seed, DHS, De-hulled seed; Score,0= not sticky, 10= stickiness similar to commercial soya natto

[2] Means + standard deviation (n=3

#, 0.2 mg 100 g^{-1} of inoculum level, pressure cooked lupin; ‡, ISC, pressure cooked lupin; †, ISC, 0.2 mg 100 $^{g-1}$

Table 3.6 Ammonical odour of lupin natto as detected and scored by evaluators using Visual analogue scale (VAS) questionnaire over the fermentation period; effect of bacterial strain, inoculum level and seed pre-cooking

Fermentation (h)	Inoculum type #				Inoculums level(CFU g^{-1}) ‡						Processing method/cooking †			
	ATCC 15245		ATCC 6633		102-4		104-6		106-8		Boiling		Pressure cooking	
	WS	DHS	WS	DHS	WS	DHS	WS	DHS	WS	DHS	WS	DHS	WS	DHS
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0.5	0	0	0	0	0.5	0	0	0	0	0
12	0.5	0	0.5	0.5	0	0	0	0	0.5	0.5	0.5	0.5	0	0
18	1	0.5	1	2	0	0.5	0.5	0.5	1	1	2	1	0.5	0
24	2	1	3	5	0.5	0.5	0.5	0.5	4	4	3	3	0.5	0.5
30	2	3	5	7	0.5	1	1	1	5	5	5	7	1	1
36	3	4	6	8	0.5	2	3	3	7	7	6	7	3	3
42	4	8	6	8	1	4	5	5	7	6	8	6	5	5
48	6	7	8	9	4	5	5	5	8	7	9	8	5	6
54	9	10	9	10	5	6	8	8	8	8	9	8	6	8
60	10	10	10	10	6	6	8	8	10	10	10	9	6	8

WS, Whole seed, DHS, De-hulled seed; Score,0= no ammonia smell, 10= extremely high ammonia smell
ISC, Indonesian commercial Starter; USC, USA commercial stater; WS, Whole seed, DHS, De-hulled seed
Score: 0, not sticky, 10, stickiness similar to commercial soybean natto
#, 0.2 mg 100 g^{-1} of inoculum level, pressure cooked lupin; ‡, ISC, pressure cooked lupin; †, IC

3.4.3 Effect of processing methods on chemical and physical properties of lupin tempeh

Statistical analysis showed that different processing methods and source of lupin significantly affect ($P < 0.05$) the ammonia content, total plate count and hardness but processing method did not significantly affect the hardness and moisture (Table 3.7). Source of lupin significantly affected ($P < 0.05$) total mould count, ammonia content and moisture but not significantly affected by the pH and hardness. According to post hoc test (Bonferroni) processing methods (boiled and pressure cooked) and source of lupin (WS and DHS) had significant effect *($P < 0.05$)* on ammonia content but there was no significant effect on pH, moisture and hardness. Also, in pair, wise comparison source of lupin had a significant effect on total count, ammonia content and moisture. However, pH and hardness were not significantly affected by source. Further, processing methods significantly affected on pH, moisture and hardness (Table 3.7). Steinkraus et al. (1960) showed that de-hulling of the beans is important for growth of the mould on the cotyledons. Present results agree with the above concept and tempeh prepared from de-hulled seed lupin had significantly higher total mould count and high ammonia content. Lupin has a thick hull portion (seed coat) and it is hard for mould to grow on the hull. Therefore, growth rate on whole seed is reduced compared to dehulled seed. this may be the reason for dehulled lupin tempeh had a higher mould count and ammonia content compared whole seed. Also, as shown in Table 3.7 processing method pressure cooking (121 °C 15 psi^{-1}) had significant effect on total mould count and ammonia content. This could be due to destroying competitive bacteria by high temperature by the pressure cooking as it involves high temperature (121 °C 15 psi^{-1}) destroy anti-nutritional factors

and release some of the nutrients required (Steinkraus, 1983). Jurus and Sunberg (1976) reported that the penetration and growth of the hyphae, which occurs only between the cylindrical cotyledons cells. Further, the changes occurring during fermentation resulted from deep penetration of enzymes via diffusion from the outer surface layer to inner part of the sample. As shown in Table 3.7 moisture content of mature lupin tempeh ranged from (53.7-64.2%) and these values are smaller than moisture values (68.0-69.4%) reported by Agosin (1989). Reason for the difference may be due to experimental procedure differences, values in present study reported for whole seed and dehulled lupin instead Agosin (1989) have used dehulled grits. Similar values were reported in a study of evaluation of quality and sensory of lupin tempeh prepared from various particle size of lupin seeds (Priyatni, 2013).

pH constantly increased as the fermentation proceeded, pH of tempeh prepared from boiled whole seed lupin was 7.15 at 48 h fermentation. Similar results were reported by Agosin (1989). However, tempeh prepared from pressure cooking processing method had lower pH compared to tempeh boiled. The difference in pH was probably due to the difference in the method of preparation variety of soybean used, and also length of fermentation since the production of ammonia during fermentation.

Observation showed that the firmness (Table 3.7) of lupin tempeh prepared from boiling was higher than of lupin tempeh prepared from pressure cooked lupin bean. This may be to high temperature and pressure softened the cell wall better than boiling. However, as explained in Priyatni (2013) with mycelia growth there will be a strong network of mycelia among lupin bean. Further, these firmness values are comparable with our findings. There was no significant ($P > 0.05$) difference between firmness of whole seed tempeh

and dehulled tempeh (Table 3.7). To ensure the growth of mycelia on the lupin beans and gap among the beans, it requires minimum 24 h (Handoyo & Morita,2006). Due to fermentation of mycelia and strong network among lupin beans firmness reduced with fermentation time. Different processing methods had affected significantly (*P* > 0.05) on firmness as the pressure (Table 3.7).

cooking (121 °C 15 psi^{-1}), which could have made the microstructure of the beans more penetrable to the mould, thereby affecting the firmness of the tempeh.

Table 3.7 The effect of processing method (seed pre-cooking) on physical and chemical properties of lupin tempeh[1]

Nutrient component & source		BL	PCL	Pro_Method	Source	P x S
Moisture %	WS	61.10±0.32[a]	64.20±0.32[a]	NS	P=0.005	P=0.001
	DHS	53.70±0.18[b]	55.23±2.02[b]			
pH	WS	7.05±0.21[a]	6.90±0.28[a]	NS	NS	NS
	DHS	6.70±0.28[a]	6.45±0.21[a]			
Firmness (kg)	WS	26.42±3.16[a]	20.90±2.05[b]	NS	P=0.042	NS
	DHS	21.67±2.14[a]	18.97±0.93[b]			
Total mould count (cfu/g) $10x^4$	WS	3.25±0.49[a]	1.95±0.78[a]	P=0.002	NS	NS
	DHS	6.20±0.99[a]	3.75±0.64[a]			
Ammonia content mg 100 g^{-1}	WS	205.36±11.62[a]	265.49±3.92[c]	NS	P=0.006	NS
	DHS	252.55±10.25[b]	295.33±12.71[d]			

BL, boiled lupin (60 min); PCL, pressure cooked lupin (60 min) ; WS, whole seed; DHS, de-hulled seed [a-d] Means (SD) of values , means within the same row with different letters indicate the significantly difference values

[1] lupin seeds samples soaked in water at room temperature for 20h, fermented with Indonesian commercial starter (ISC) for 48 h at 34 °C

3.4.4 Effect of Processing (seed pre-cooking) methods on chemical and physical properties of lupin natto.

Table 3.8 represents the characteristic of lupin natto prepared using different seed pre-cooking methods (boiling and pressure cooking) and lupin seed source (WS and DHS). Statistical analysis showed that different processing methods and source did not have significant ($P > 0.05$) effect on chemical and physical properties of lupin natto (Table 3.8). However, according to between subject effect, processing methods had significant effect ($P < 0.05$) on all chemical and physical properties while source of lupin had significant ($P < 0.05$) effect on pH, hardness and ammonia content but there was no significant effect on moisture and total bacteria count (Table 3.8). In pairwise comparison test seed processing methods had significant effect ($P < 0.05$) on all physical and chemical properties including moisture, pH, firmness, ammonia content and total plate count of lupin natto. However, lupin seed source (WS & DHS) significantly ($P < 0.05$) affected only on characteristic pH, firmness and ammonia content whereas moisture and total plate count were not affected significantly.

There are many factors affecting the quality of natto and tempeh such as seed pre-soaking, seed processing methods (boiling, pressure cooking), seed cultivar, microbial strain and fermentation time (Wei et al., 2001). Soybean which is small, round and uniform size with clear hila and smooth seed coat produces best quality natto. Small soybean has high water binding capacity, high carbohydrate content and low oil content. Because soluble sugars serve as an initial carbon and energy source for *B. natto* their high carbohydrate content promotes microbial growth and make the finished product taste sweeter.

Hard steamed beans could produce unfavourable ammonical flavours in natto but high water –absorbing capacity yielding soft steamed beans was desired (Taira, 1990b). Ohta (1986) reported that soybean with high carbohydrate content produced less ammonia than soybean with low carbohydrates. Moisture content is another criterion related to water–holding capacity. The range of moisture content (58.9-60.5%) of steamed beans was similar to the range of moisture content (58.2-61.6%) of steamed soybean reported by (Taira et al 1987). This could be due to high water holding capacity of lupin (Sujak, Kotlarz, & Strobel, 2006)

Table 3.8 The effect of processing method on physical and chemical properties of lupin natto[1]

Nutrient component & source		RL	PCL	Pro_Metd	Source	P x S
Moisture %	WS	61.15±1.77a	59.15±0.35b	P=0.045	NS	NS
	DHS	61.35±0.64a	58.12±1.72b			
pH	WS	7.91+0.12a	8.17+0.25c	P=0.003	P=0.009	P=0.07
	DHS	6.93+0.11b	6.45±0.21[a]			
Firmness (kg)	WS	37.03+1.46a	32.51+2.15c	P=0.049	P=0.019	NS
	DHS	31.88+2.43b	29.45+0.71d			
Total plate count (CFU g^{-1}) $10x^8$	WS	4.45+0.35a	4.95+0.21b	P=0.010	NS	NS
	DHS	4.00+0.14a	5.20+0.28b			
Ammonia content mg 100 g^{-1}	WS	271.16+4.25a	292.99+5.48c	P=0.003	P=0.003	NS
	DHS	252.55±10.25[b]	270.96+8.16d			

BL, 60 min boiled lupin; PCL, 30 min pressure cooked lupin (121°C/15psi); WS, whole seed; DHS, dehulled seed; [a-d] Means of triplicates, means within the same row with different letter indicates significantly different values; [1] Lupin seed samples soaked in water at room temperature for 20h, fermented with *B. natto* (ATCC 15245) for 48 h at 37 °C

3.4.5 The effect of microbial strain on physical and chemical properties of lupin natto

The results presented in Table 3.9 show that the effect of bacteria strains on physical and chemical properties of lupin natto. *B. subtilis* strains ATCC 15245 and ATCC 6633 grew well on the plate count agar (PCA), when we checked for viability of the cultures upon receipt. Therefore, they were inoculated onto 60 min pressure cooked (121 °C/15 psi) whole and dehulled lupin seed and ferment at 38 °C for 24 h and the moisture, pH, hardness, ammonia content and total plate count were determined. According to (Matsumoto et al.,1993) original natto (from soybean) fermentation proceeded in good condition inoculation of 10^{-4}-10^{-6} CFU g^{-1} or 40 °C, 10^{-2}-10^{-4} CFU g^{-1} at 35 °C of the appearance, colour and hardness for 18-20 h of fermentation (Matsumoto et al.,1993). According to our preliminary study data, initial bacterial population of the samples ranged 10^{6-} CFU $g^{-1}$and population of the bacteria in the final product ranged 10^{8-9} CFU g^{-1}. Bacteria strain and source had significant overall/ main effect on physical and chemical characteristic of lupin natto. Microbial strain had a significant ($P < 0.05$) effect on ammonia content, total plate count, pH, hardness and moisture. However, source had a significant effect on pH, ammonia content and hardness but had no significant on total plate count and moisture. ATCC 15245 had high population (7.05-9.10 x 10^{8} CFU g^{-1}) compared to ATCC 6633 (3.92-4.12 x 10^{8} CFU g^{-1}). Also, ammonia content of lupin natto prepared from ATCC 15245 was higher (Table 3.9) than ATCC 6633 lupin natto.

Table.3.9 The effect of microbial strain on physical and chemical properties of lupin natto[1]

Properties	Source	ATCC 15245 (M1)	ATCC 6633 (M2)	Micro_strain	Source	M1 x M2
Moisture (%)	WS	63.41±1.80a	59.19±0.85b	P=0.045	NS	NS
	DHS	60.94±0.83a	56.66±1.73b			
pH	WS	8.26±0.13a	7.82±0.21c	P=0.003	0.009	P=0.028
	DHS	8.0±0.10b	6.79±0.20d			
Firmness (kg)	WS	33.71±0.99a	38.92±1,91c	P=0.049	0.019	NS
	DHS	27.09±3.96b	33.66±2.90d			
Total plate count	WS	7.05±0.61a	3.92±0.76b	P=0.010	NS	NS
	DHS	9.10±0.96a	4.12±1.64b			
Ammonia content (mg 100 g^{-1})	WS	294.07±1.94a	257.79±5.03c	P=0.003	P=0.003	NS
	DHS	266.76±8.21b	238.01±6.56d			

ATCC15245, lupin natto samples prepared from *B. subtilis natto* ATCC 15245 lupin; PCL, lupin natto samples prepared from B. subtilis natto ATCC 6633; WS, whole seed; DHS, de-hulled seed

[a-d] Means (SD) of duplicates. Means within the same row with different letters indicate the significantly difference values

1 lupin natto prepared from seeds samples soaked in water at room temperature for 20 h, from pressure cooked lupin for 24 h at 37 °C

3.4.6 Effect of microbial strain on physical and chemical characteristic of lupin tempeh

Table 3.10 presents the effect of microbial strain on physical and chemical characteristic lupin tempeh. There was a significant effect of microbial strain (Commercial starter *R. oligosporus* from USA and Commercial starter from Indonesia) and source (whole seed and dehulled seed) on physical and chemical characteristic of lupin tempeh. However, there was no significant of microbial strain x source.

Also, in pair wise comparisons, source whole seed and dehulled seed had significant difference on moisture, hardness and ammonia content. There was no significant (P > 0.05) effect on pH and total plate count. Further, in a pair wise comparison *R. oligosporus* commercial USA starter culture and commercial Indonesian starter culture had a significant different effect on ammonia content and total plate count. However, microbial strain had no significant (P > 0.05) effect on moisture, pH and hardness.

Table 3.10 The effect of microbial strain on physical and chemical properties of lupin tempeh

Properties	Source	USC	ISC	Micro_strain	source	T x S
Moisture (%)	WS	61.40±2-12[a]	66.70±1.70[a]	NS	P=0.005	NS
	DHS	56.20±2.40[b]	62.32±1.89[b]			
pH	WS	7.35±0.21[a]	6.70±0.49[a]	NS	NS	NS
	DHS	6.93±0.14[a]	6.30±0.14[a]			
Firmness (kg)	WS	27.19±2.07[a]	23.73±0.88[a]	NS	P=0.015	NS
	DHS	21.67±2.14[b]	20.150.47±[b]			
Total mould count	WS	2.70±0.14[a]	5.29±0.47[b]	P=0.003	NS	P=0.016
	DHS	3.60±0.14[a]	4.15±0.49[b]			
Ammonia content (mg 100 g^{-1})	WS	202.77±4.07[a]	267.32±1.90[c]	P=0.001	P=0.001	NS
	DHS	252.28±5.72[b]	295.05±9.49[d]			

USC, USA commercial starter (cultures for health); ISC, Indonesian commercial starter culture; WS, whole seed; DHS, de-hulled seed

[a-d] Means (SD) of duplicates. Means within the same row with different letters indicate the significantly difference values

[1] lupin natto prepared from seeds samples soaked in water at room temperature for 20 h, pressure cooked (121 °C 15 psi^{-1}) for 48 h at 37 °C

3.4.7 The effect of bacteria strain and pH on lupin natto during fermentation period

Previous research findings indicate that, optimum time for producing of soybean natto ranged 6 h (Sakurai, 1960) – 20 h (Beucchat,1983; Ohta,1986, Maruo & Yoshika, 1989). The pH values of lupin natto prepared from bacterial strain ATCC 15245 and ATCC6633 at 0 h (pressure cooked WS and DHS lupin at 34 °C) was 5.8, 5.93, 6.0 and 6.1, respectively. As presented in Figure 3.10, pH value increased with fermentation time. Using dehulled seed lupin and ATCC 15245 strain, natto products showed rapid increase in pH value during 10-12 h of fermentation (pH, 6.6). The lupin natto prepared from whole seed, ATCC 6633 strain had lowest pH (pH, 5.8-8.1) throughout the fermentation period (0-48 h). As presents in Figure 3.10, lupin natto prepared from whole seed had lower pH value compared to dehulled lupin seed. Also, microbial strain ATCC 15245 produced lupin natto with high pH value compared to ATCC 6633. This indicates that ATCC 15245 strain grow better on lupin source than ATCC 6633 strain. Also, it indicates that source of lupin (WS & DHS) and stain of *B. natto* affect the lupin natto.

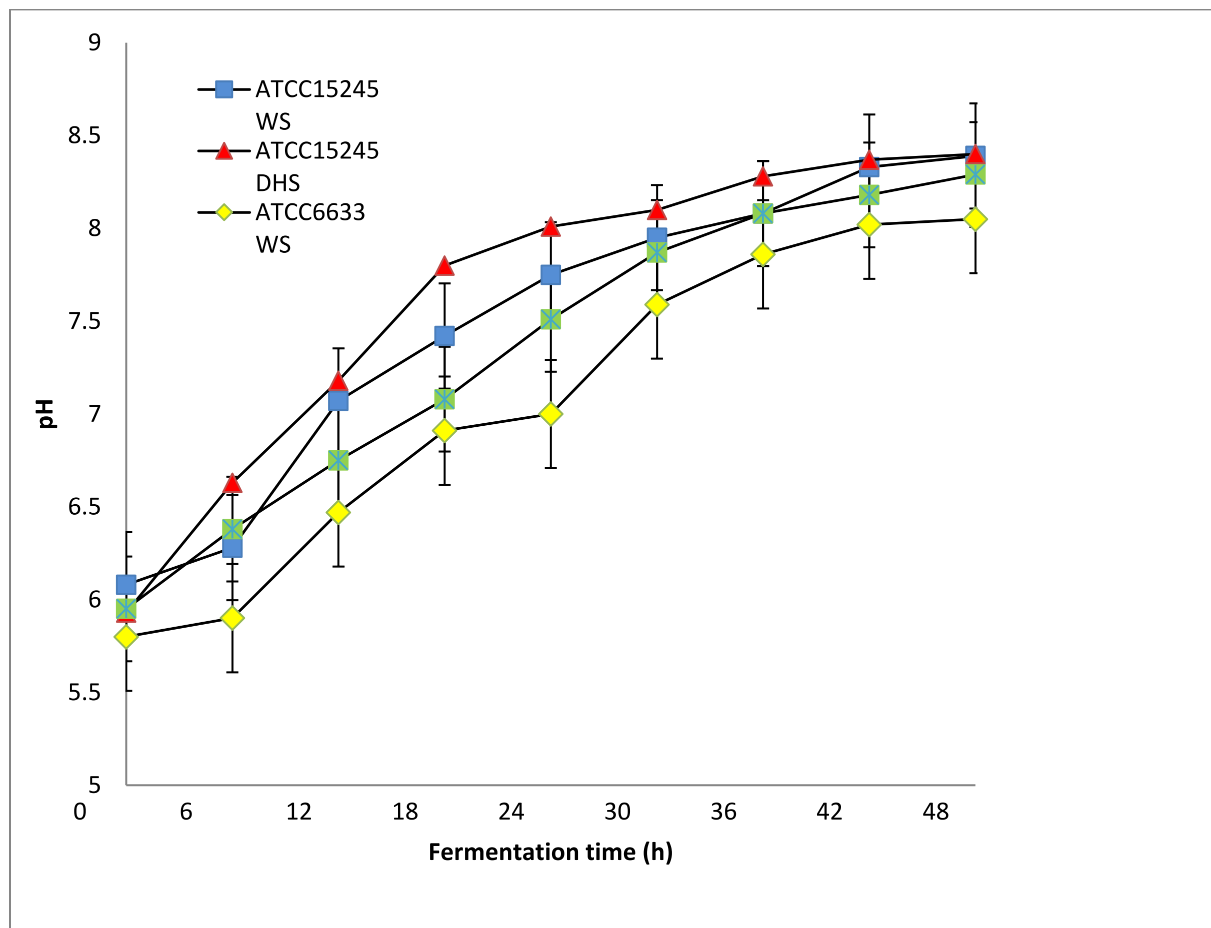


Figure 3.10 Effect of source of lupin (WS & DHS), bacteria strain (ATCC 6633 & ATCC 15245) and fermentation time (0-48 h) on pH value of lupin natto.

3.4.8 Change of pH and temperature of lupin natto and tempeh during fermentation time

As shown in Figure 3.10, initial (0 h) temperature of lupin tempeh (pressure cooked beans, ISC) was 34.1 ºC and final temperature reached up to 37.5 ºC for WS. Similarly, DHS lupin tempeh ranged from 33.9 ºC-37.6 ºC with respect to fermentation time. There was no significant temperature difference between ($P < 0.05$) WS and DHS lupin tempeh. According to fermentation time lupin natto temperature ranged from 36.1 ºC to 40.9 ºC in WS and from 36.0 ºC to 41.1 ºC in DHS. Initial pH of lupin tempeh was 4.10 (WS) and 4.25 (DHS). Final pH reached up to 8.15 (WS) and 8.45 (DHS), respectively. Initial pH of lupin natto (pressure cooked lupin, *B. subtilis* ATCC15245) was 6.0 (WS) and 6.15 (DHS) and final pH values were 8.34 (WS) and 8.44 (DHS) respectively. These pH increases were corresponded with temperature increase in the products. Similarly, ammonia content increase with temperature and pH increase (Figure 3.11). Due to biochemical changes during fermentation, (action of proteolytic enzyme) ammonia is produced as a by-product. Formation of ammonia increases the pH in the samples. The increase in pH affects the structure of proteins and this may suppress the action of fermentation enzymes. Because of that mould growth reduces and finally, which leads it to end of mycelium growth. As result of ammonia increase product gets spoiled. Therefore, ammonical odour increases with the pH increase.

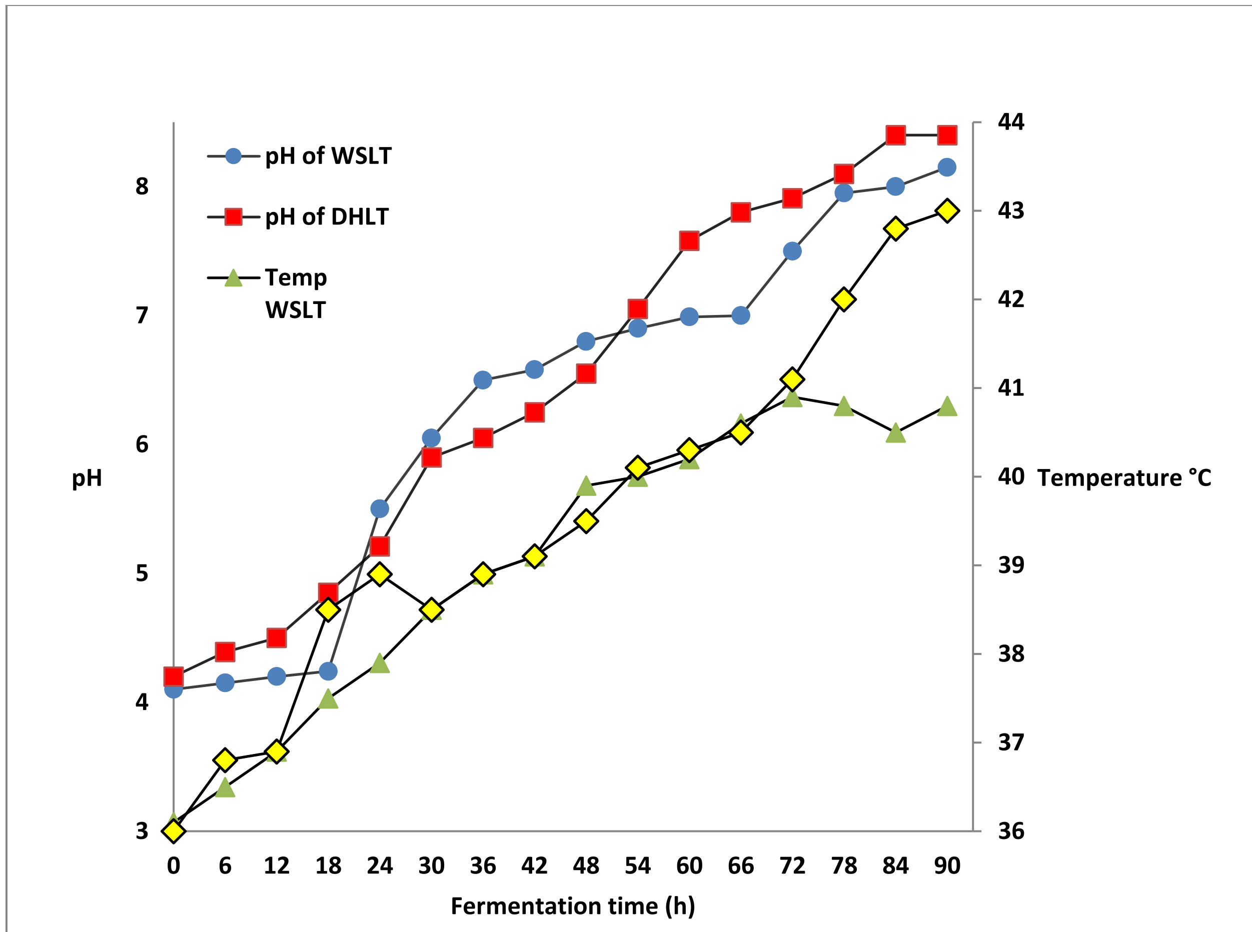


Figure 3.11 pH and temperature changes with fermentation time

3.5 Conclusion

This study determined the characteristics of lupin tempeh prepare from whole lupin seed and dehulled lupin seed fermented with two *R. oligosporus* strains separately. Also, results indicated that dehulled lupin seed and *R. oligosporus* strain from Indonesia may be better raw materials to prepare best quality lupin tempeh.

Moreover, laboratory development of lupin natto (natto analogue from lupin) was prepared for first the first time. There is no literature on production, characterizing and analysing of lupin natto. Therefore, lupin may be used as a suitable substitute for Asian fermented food industry as a raw martial for natto and tempeh production.

CHAPTER 4

EFFECT OF PROCESSING AND DIFFERENT FERMENTATION APPROACHES ON NUTRITIONAL COMPOSITION AND DIETARY FIBRE PROFILE OF AUSTRALIAN SWEET LUPIN (*LUPINUS ANGUSTIFOLIUS*)

4.0 Connecting statement

In the previous chapter, it was presented that natto and tempeh analogues can be successfully produced from lupin. In this chapter, the novel fermented lupin foods which were explained in previous chapter were analysed for the biochemical changes of macronutrients and micronutrients (nutritional composition) that occurs during fermentation and how the fermentation can be utilised to improve nutritional value of lupin.

4.1 Introduction

The demand for legume seed has increased globally due to their use as a human food (Sinclair & Vadez, 2012). The food industry and health food producers are constantly on the lookout for functional food ingredients of high nutritional and functional value combined with low cost (Jayasena et al, 2004). Australian sweet lupin (ASL) is a such kind of food ingredient and has been attracted by food industry and health food producers due to its importance as a functional food. The functional value of a food can be defined as having a potential health-promoting effect, or in reducing the amount of known adverse components (Martínez-Villaluenga, Frías, & Vidal-Valverde, 2006). Monteiro et al. (2014) indicated that lupin flours of *L. albus* and *L. angustifolius* contained 36.3 and 36.9 g 100 g^{-1} respectively. Also, fat and dietary fibre content were 8.37: 4.39 and 9.11: 23.41 g 100 g^{-1}. Further, as described (Johnson et al, 2017) average lupin seed protein content *L. albus,* 38.2; *L. angustifolius, 33.9 L. luteus, 42.2 and L. mutabilis,* 43.3. (g 100 g^{-1} db). Compared to lupin, soybean seed contains 37.0 g 100 g-1 with soybean meal 44 g 100 g^{-1} db. Australia is the main lupin seed producer in the world and annual production was 625,600 tonnes in 2014 which is 85% of the world production (FAOSTAT, 2014). Unfortunately, it has been mainly used as an animal feed in Australia until the Food Standard Australia and New Zealand declared it “fit for human consumption” in 1987 (Belski et al., 2011). However, as a result of recent scientific research identifying lupin’s health benefits, it is now being more widely considered as a human food source (Clark, Clark, & Johnson, 2002).

During tempeh producing from dehulled soybean and lupin bean, protein increased from 42.5 g 100 g^{-1} db. to 44.6 g 100 g^{-1} db. in lupin and from 45.4-53.8 g 100 g^{-1} db. Also, fat content was increased from 8.5 to 8.7 (g 100 g^{-1} db.) in lupin and 22.2 – 22.6 (g 100 g^{-1} db.) (Fudiyansyah et al., 1995a). Essentially lupin natto has not been developed earlier and there hasn't been any published literature up to date.

Further, the changes of dietary fibre profile during *B. subtilis* and *R. oligosporus* fermentation of lupin have never been reported. Therefore, the aims of this study were to investigate changes in nutritional composition and dietary fibre profile

(a) between raw whole seed, dehulled seed, soaked and boiled lupin seed

(b) during fermentation of whole (WSL) and dehulled lupin (DHSL) by the bacteria *B subtilis* to produce a natto analogue and mould *R. oligosporus* to produce a tempeh analogue.

4.2 Materials and methods

4.2.1 Materials

Ethanol 96% v/v, ethanol 78% v/v, acetone, reagent grade, 2M Acetic acid solution, 0.75M tris base solution, 150 mM HCl solution, celite and sodium maleate buffer and all other chemicals were purchased from Sigma Aldrich, New South Wales, Australia. Pancreatic α-amylase, stock amyloglucodase and protease were purchased from Megazyme Co. Bray, Wicklow, Ireland.

4.2.2 Methods

4.2.2.1 Preparation of fermented lupin tempeh

Lupin tempeh was prepared as described in section 3.3.2.6 using WS and DHS.

4.2.2.2 Preparation of lupin natto

Lupin natto samples for this study were prepared as described in 3.3.3.5 using WS and DHS.

4.2.2.3 Moisture, proximate composition and other chemical analyses

All analyses of samples were conducted in at least triplicate. Proximate composition was determined using AOAC methods (AOAC, 2008). Moisture content was determined using AOAC oven drying method 925.10 (D-91126 Schwabach FRG, Germany. Ash was determined as per the AOAC method 923.03 (AOAC 2008). Protein was determined by Kjeldahl AOAC method 920.87 (Nx5.4) (Mosse, 1990). Crude fat was determined by petroleum ether extraction (Buchi E-816, Flawil, Switzerland) using AOAC Method 945.16 (AOAC 2008).

4.2.2.3.1 Moisture and ash content

Moisture analysis was performed on fresh samples and they were weighed in an uncovered dry moisture dish and dried in an oven (D-91126 Schwabach FRG, Germany) at 105 °C. The samples were removed from the oven every 3 h and cooled in a desiccator for 2 h to reach room temperature. The samples were then weighed until a constant weight was achieved. Once a constant weight was achieved, the moisture content was calculated from weight loss g 100 g^{-1} as is (AOAC, 2008). Ash was determined by ashing at 550 °C in a furnace (Thermoline 48000, Dubuque, Iowa, USA) until constant weight overnight as per the AOAC method 923.03 (AOAC 2008). The crucible was cooled in desiccator for 1 h and weighed it to nearest 0.1 mg. Ash was determined by subtracting tare weight (weight of dried crucible and celite). (See figure 4.3 f or the details)

4.2.2.3.2 Protein content

Protein content of the samples was determined on the freeze-dried samples by Kjeldahl method (950.36) AOAC (2000). The analysis was carried out using a Tecator Digestion System and a Kjeltec 1030 auto analyser. Weighed samples were placed in a test tube, followed by addition of a catalyst (Kjeltab $Na_2S_2O_8/CuSO_4$), a glass bead, 10 mL of digestion acid (conc. sulphuric acid) and 4 mL of hydrogen peroxide. The tubes were immediately placed into pre-heat digester set at 420 ^{0}C. The samples were digested until the solutions became clear or pale yellow. The digested samples were then cooled down to room temperature and then 75 mL of distilled water was added slowly. A receiver flask containing 25 mL of boric acid and indicator (bromocresol green solution and methyl red solution) was placed under the condenser outlet of Kjeltec machine and 40% NaOH was poured into the digested samples. Subsequently, the steam distillation was continued until 125 mL of final volume was collected in to the receiver flask. The ammonia content trapped in the boric acid was titrated against 0.1M hydrochloric acid until a grey endpoint was reached. The amount of titrate is equivalent to the amount of ammonia present in the samples (% N). The protein content (g/100g db) was calculated by multiplication of % N with a conversion factor (f) of 5.4 for lupin (Mosse, 1990).

4.2.2.3.3 Oil content

Oil content of the samples was determined on the freeze-dried samples by a modification to the AOAC method 963.15 (AOAC 2008) using a Buchi E-816 SOX extraction unit. Weighed samples were placed into corresponding thimbles and subsequently were placed above the pre-weighed extraction cups containing glass beads (We) in the extraction unit. The samples were extracted by petroleum ether for 90 min. Following the extraction, the solvent remaining in the extraction cup was evaporated and the cups containing the oil extracted were then dried in an oven at 105 °C for 24 h. The dried extraction cups were weighed, and oil content was calculated as g 100 g^{-1} db.

4.2.2.3.4 Determination of *in vitro* protein digestibility (IVPD)

The *In-vitro* protein digestibility (IVPD) of all samples was measured according to the modified method of pepsin pancreatin digestion method (Akeson & Stahmann, 1964; Maliwal, 1983). Samples (1.0 g) were incubated at 37 °C with 0.75 mg pepsin (in 7.5 mL of 0.1N HCl, pH 2; 2500 units mg^{-1} activity, Chem Supply, Gillman, SA, Australia) and they were incubated for 16 h at 37 °C. The undigested protein in 5 mL of digest was then precipitated by addition of 25 mL of 10 % TCA and centrifuged for 30 min at 1000 x g. Nitrogen in the supernatant was determined using the Kjeldahl digestion and distillation method as previously used in this chapter. *In-vitro* protein digestibility (IVPD) (%) was calculated according to equation 1

$$\text{IVPD } \% = \left(\frac{\text{Nitrogen in the supernatant g}}{\text{Total nitrogen in the sample g}}\right) \times 100 \quad \text{...... Equation 1}$$

4.2.2.3.5 Analysis of dietary fibre-Integrated total dietary fibre assay procedure

AOAC method 2011.25 (AOAC, 2011) was followed in this procedure. The lyophilized, milled sample was weighed (1.000 ± 0.005 g) accurately in to 250 mL wide mouth bottles. Then 1.0 mL of ethanol and 40 mL of pancreatic α-amylase/amyloglucosidase mixture was added to each bottle. Bottles were incubated at 37 °C for 16 h at 150 rpm in a shaking water bath. Then 3.0 mL of 0.75 M tris base solution was added and then the samples were further incubated for 20 min at 95-100 °C. Samples were then cooled 60 °C and protease solution (1 mL) was added and incubated at 60 °C for 30 min. 2M acetic acid (4.0 mL) was then added to make the final pH 4.3 crucibles containing celite were tared to nearest 0.1 mg. The bed of celite® in the crucibles was wetted and redistributed using 15 ml of 78 % ethanol (v/v). Suction was applied to the crucibles to form an even mat. The enzyme digest was filtered through the crucibles using vacuum. The incubation bottles were rinsed with 10 mL 60 °C deionised water using a wash bottle and rubber spatula was used to dislodge all particles from the wall of the container. The suspension was transferred to the crucible. Combined filtrate and washings were collected, and volume was adjusted to 70 mL and retained this for determination of SDF. The residue was washed with two 15 mL portions of 78 % ethanol, 95 % ethanol and acetone. Washing was discarded. Then the crucible containing residue was dried overnight in the oven at 105 °C. After that crucible was cooled in desiccators for 1 h and weighed it to nearest 0.1 mg. To obtain residue mass, subtracted tare weight (weight of dried crucible and celite (AOAC method 2011.25).

IDF was calculated as shown in Equation 5.

Blank

(B)determination=$[(BR_1+BR_2)\div2]-P_B-P_A$

Where:

BR1 and BR2= residue mass (mg) for duplicates blank determination respectively.

PB and PA =mass (mg) of protein and ash respectively, determined on first and second blank residue

Insoluble Dietary Fibre (IDF)= $\frac{(R_1+R_2\div2)-P_B-P_A-B}{(M_1+M_2\div2)} \times 100$------------Equation 2

Where,

R1= residue mass 1 from M1 in mg. R2=residue mass 2 from M2 in mg; M1=test portion mass 1 in g; M2=test portion mass 2 in g; PA=ash mass from R1in mg; PB=protein mass from R2 in mg, IDF %= IDF (mg 100 g-1)/1000

Determination of Soluble Dietary Fibre Precipitate (SDF)

The filtrate from each sample (approx. 70 mL) was pre-heated to 60 °C and pre-heated (60 °C) 280 mL of 95 % ethanol (v/v) was added and mixed thoroughly. The precipitate was allowed to form at room temperature for 60 min. Then the crucibles containing celite® was tared to the nearest 0.1 mg. The bed of celite® in crucible was wet and redistributed using 15 mL of 78% (v/v) ethanol from wash bottle. Suction was applied to crucibles to draw celite onto the fritted glass as an even mat. The enzyme digest was filtered through the crucibles using vacuum. The all remaining particles quantitatively transferred to crucible using a wash bottle with 78% (v/v) ethanol. Filtrate and washings were retained proceed to SDFS analysis (further studies). Residue was subsequently washed with two 15 mL portions of 78% (v/v) ethanol, 95 % ethanol and acetone. Then the crucible containing residue was dried overnight in the oven at 105 C°. After that crucible was cooled in desiccators for 1 h and weighed it to nearest 0.1 mg. To obtain residue mass, subtracted tare weight (weight of dried crucible and celite). SDF was calculated as shown in Equation 6.

$$\text{Blank (B) determination} = \frac{(BR_1 + BR_2)}{2} - P_B - P_A$$

Where:

BR_1 and BR_2= residue mass (mg) for duplicates blank determination respectively.

P_B and P_A =mass (mg) of protein and ash respectively, determined on first and second blank residue

Soluble Dietary Fibre

$$SDFP = \frac{[(R_1 + R_2 \div 2) - P_B - P_A - B] \times 100}{(M_1+M_2)/2} \quad \text{..........................Equation 3}$$

Where R1= residue mass 1 from M1 in mg.

R2=residue mass 2 from M2 in mg.

M1=test portion mass 1 in g, M2=test portion mass 2 in g.

PA=ash mass from R1, in mg: PB=protein mass from R2 in mg.

SDFP %= SDFP (mg 100 g^{-1}) 1000

Protein and ash determination

The residue from one crucible (refer to section 4.3.2.5) was analysed for protein, and the second residue of the duplicate is analysed for ash. Protein analysis on residue was performed using Kjeldahl method as described in section 4.4.2.7. Conversion factor (5.4) was used for lupin and lupin products to calculate mg of protein. The second residue was incinerated for ash analysis as described in section 4.4.2.6. (Megazyme, 2015).

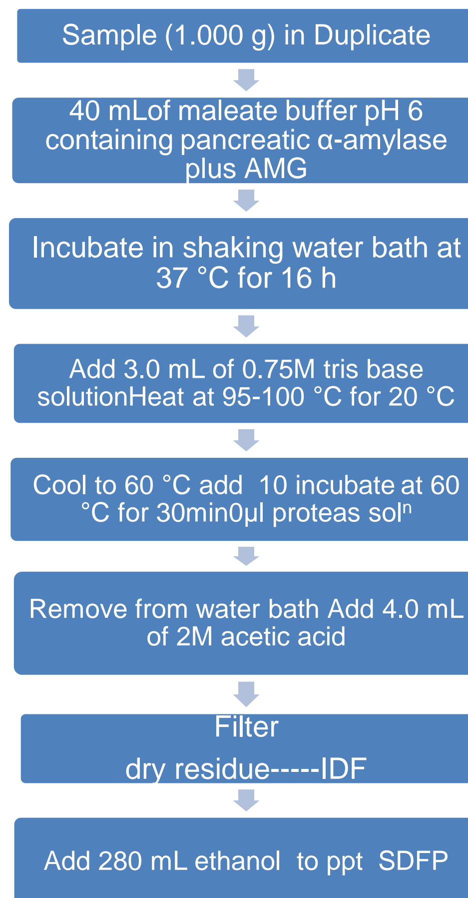


Figure 4.1 Analytical scheme for the determination of insoluble dietary fibre (IDF) and soluble dietary fibre precipitate in ethanol (SDF)

4.3 Statistical Analysis

Data are presented as mean values with standard deviation. Repeated measures ANOVA with within samples factors (for the time effect), between sample factors (for sources; WS and DHS) and their interaction (time x source) was used. When main effects or interaction were significant, individual means were compared by Bonferroni test. $P < 0.05$ was considered as significant. IBM SPSS statistics V.24 (IBM Corp. NY, USA) was used for all analyses.

4.4 Results and Discussion

4.4.1 The effect of processing prior to fermentation and seed source on proximate composition

As shown in Table 4.1 processing stages (soaking, cooking, dehulling) and source of lupin (whole seed and de-hulled seed) had significant effect ($P < 0.05$) and interaction on protein content. Protein percentage of dehulled lupin seed was higher compared to whole seed to at all three processing stages. Compared to the present results Fudiyansyah et al. (1995a) indicated increase in protein content from raw dehulled lupin to cooked. A similar effect was recorded on cooking during tempeh preparation from soybeans (van der Riet, Wight, Cilliers, & Datel, 1987). The protein content of the raw dehulled lupin was slightly lower than previously reported at 41.1 g 100 g^{-1} db. (Villarino et al., 2016), 41.8 g 100 g^{-1} db (Hall et al., 2005) and 41.2 g 100 g^{-1} (Saez et al., 2015). In contrast, it was indicated by (Asiedu, Nilsen, Lie, & Lied, 1993) and reported that processing has no marked effect on crude protein and fat of sorghum and maize. The reason for the differences in reported data with present data found in this experiment could be due to variety/cultivar and also these dissimilarities may occur as a result of the nature of the growing conditions and soil type. Further, the protein content of samples tested closely resembled that of soybean (30-40 g 100 g^{-1} db.) components except for crude fibre (Saez et al., 2015). According to their results, de-hulled soaked lupin had 42.4 g 100 g-1 db. and cooked whole seed had 33.4 g 100 g^{-1} db. of protein. As lupin contained comparable protein content to soybean it is suitable for preparing traditional Asian fermented legume foods tempeh and natto.

Further, Table 4.1 shows the effect of processing type and source of lupin on fat content of ASL. There was a significant effect ($P < 0.05$) of processing type and source on fat content on fat content with the de-hulled seed having significantly higher ($P < 0.05$) content than whole seed. The fat content of whole seed and de-hulled seed ranged from 5.6-8.0 g 100 g^{-1} db which agrees with (Hall et al., 2005). while slightly lower than 7.8-8.8 g 100 g^{-1} db. (Villarino et al., 2016). However, there was no interaction in processing methods × source. Source of lupin had significant effect ($P < 0.05$) on ash content of ASL. There was no significant effect of processing method on ash content of ASL. Ash content of the samples of ASL ranged from 2.8-3.8 g 100 g^{-1} db with significant effect ($P < 0.05$) of source. There was no statistically significant interaction between processing method × source. The moisture content of samples ranged from 10.5 -65.6 g 100 g^{-1} db with lowest ($P < 0.05$) value of whole seed raw lupin to highest value ($P < 0.05$) of cooked de-hulled lupin. There was a significant effect ($P < 0.05$) of processing type and no significant effect of source on moisture content of ASL.

Table 4.1 Proximate composition of ASL (Australian sweet lupin) † effect of different processing methods (g 100 g^{-1} db.)

Nutrient component & source		RM-ANOVA					
		RL	SL	BL	Time	Source	T x S
Protein	WS	26.4±0.36^{a}	27.6±0.59^{a}	24.9±0.31^{a}	P=0.002	P=0.001	P=0.001
	DHS	33.4±0.38^{b}	36.2±3.58^{b}	38.2±0.74^{b}			
Fat	WS	5.7±0.23^{a}	6.7±0. 08^{a}	5.5±0.07^{a}	P=0.001	P=0.004	NS
	DHS	6.2±0.17^{b}	6.9±0·07^{b}	8.0±0.28^{b}			
Ash	WS	3.7±0.45^{a}	3.1±0.18^{a}	3.1±0.19^{a}	NS	P=0.001	NS
	DHS	2.8±0.24^{b}	2.7±0.32^{b}	3.1±0.29^{b}			
Moisture (%)	WS	10.5±0.07^{a}	38.5±0.09^{c}	59.9±0.17^{e}	P=0.001	NS	P=0.002
	DHS	12.4±0.14^{b}	40.3±0.54^{d}	62.9±0.16^{f}			

†*Means of triplicate analyses ± standard deviation;* RM ANOVA, Repeated measured ANOVA with between-subject factor; NS, non significant

^{a}*Values within a row not sharing the same superscript letter are significantly different (P<0.05)*

RL, raw lupin; SL, soaked lupin; BL, boiled lupin; WS,whole seed; DHS,dehulled seed.

4.4.2 The effect of fermentation time on moisture, protein, fat and *in vitro* protein digestibility (IVPD) of lupin tempeh

As shown in Table 4.2 there was a significant main effect ($P < 0.05$) of (source whole seed lupin and de-hulled lupin), time ×source and fermentation time on protein content of lupin tempeh. Lupin tempeh which was fermented up to 24 h and 48 h had significantly higher ($P < 0.05$) protein content compared to 0 h fermented samples. The effect is comparable with previously reported around 2% increment for 45 h fermentation of lupin (Agosin et al., 1989). Moreover, according to some unpublished data in our group, lupin fermentation by *R. oligosporus* for 90 h showed increase in protein. Also, Fudiyansyah, Petterson, Bell, and Fairbrother (1995 b) demonstrated in her nutritional, chemical and sensory evaluation of lupin tempeh study, there was no difference between protein content of cooked lupin and lupin tempeh.There was a significant effect ($P < 0.05$) of fermentation time, source and fermentation time × source on fat content of lupin tempeh (Table 4.2). Lupin tempeh prepared from de-hulled seed had more protein content compared to whole seed. In pair-wise comparison test, it was shown that fat percentage increased along with fermentation time. Lupin tempeh samples fermented up to 12 h, 24 h, and 48 h had significantly higher protein content compared to 0hfermented samples. Similar results were demonstrated by Hong (2004) and protein and fat content were increased with fermentation time. Increased fat and protein content may be due to in part to the decreased oligosaccharides content after fermentation or due to increase of single (Hong et al., 2004). Fudiyansyah also recorded that decreased oligosaccharides, free sugars and other polysaccharides and increased protein and fat content during lupin tempeh (fermentation Fudiyansyah et al. 1995a).

There was significant effect of source and fermentation time on moisture content of lupin tempeh (main effect). Moisture content of lupin tempeh varied from 54.5%-66.5% and whole seed 0 h fermented sample had highest ($P < 0.05$) moisture content while dehulled 48 h sample had lowest ($P < 0.05$) moisture content. Moisture content of samples reduced when fermentation time increased. Therefore, caution must be taken to avoid drying out the product with increased fermentation time. The moisture content of 12 h, 24 h and 48 h fermented lupin tempeh were significantly decreased ($P < 0.05$) to moisture content of 0 h lupin tempeh.

Initial weight of each sample was kept constant at 200 g. There was a significant effect ($P < 0.05$) of fermentation time on sample weight of lupin tempeh. Also, sources (whole seed and de-hulled seed) showed a significant effect ($P < 0.05$) on 12, 24, 48 h fermented samples. The reduction of weight of the samples has a positive relationship with reducing moisture levels with fermentation time. Moisture content found in this study (66.5-62.2 %) is comparable with acceptable maximum moisture content of tempeh (Priatni et al., 2013). This relative moisture content is due to its high water-holding capacity (Sujak et al., 2006). Metabolic reactions of microorganisms during fermentation may also have contributed weight reduction of fermented lupin samples. i.e. Formation of CO_2 or NH_4 during fermentation as a result of metabolic reactions.

As presented in Table 4.2, *in vitro* protein digestibility (IVPD) of lupin tempeh has been significantly affected ($P < 0.05$) by fermentation time (0-48 h). However, there was no significant effect of source (WS and DHS) and source x fermentation effect on IVPD of lupin tempeh. The IVPD of lupin tempeh was increased by 10.1% during fermentation up to 48 h. The lupin tempeh which was fermented to 48 h had maximum IVPD ($P < 0.05$) while 0 h fermented samples had the lowest ($P < 0.05$). However, there was no significant difference between IVPD of lupin tempeh prepared from whole seed and dehulled seed, which means both whole seed and dehulled seed tempeh have better protein digestibility. Therefore, 48 h fermented lupin tempeh (both WS and DHS) is nutritionally important as it is the most digestible product.

Table 4.2. Australian sweet lupin tempeh: The effect of fermentation time on moisture, fat, protein and *invitro* protein digestibility (IVPD) † (g 100 g^{-1} db)

Nutrient component & source		Fermentation time (h)				RM-ANOVA		
		0	12	24	48	Time	Source	T x S
Moisture	WS	66.6±0.08[a]	65.2±0.078[b]	64.01±0.0[c]	62.2±0.10[d]	P=0.004	P=0.001	P=0.001
	DHS	59.4±0.08[f]	58.4±0.08[g]	57.4±0.08[h]	54.5±0.1[i]			
Fat	WS	5.6±0.08[a]	6.0±0.03[b]	6.1±0.15[b]	6.4±0.10[c]	P=0.001	P=0.005	P=0.002
	DHS	6.2±0.12[d]	6.9±0.042[e]	8.0±0.146e	10.3±0.11f			
Protein	WS	24.9±0.28[a]	27.4±0.09[a]	26.8±0.282[a]	30.8±0.09[b]	P=0.001	P=0.001	P=0.004
	DHS	38.2±0.22[c]	36.5±0.28[c]	36.4±0.219[c]	39.6±0.258[d]			
IVPD (%)	WS	85.9±1.58[a]	89.0±1.00[a]	91.2±0.545[b]	93.2±1.00[c]	P=0.001	NS	NS
	DHS	87.1±1.58[d]	91.9±1.00[e]	93.2±0.545[e]	96.0±1.00[e]			
Sample weight	WS	200.5±0.32[a]	195.2±0.15[b]	180.2±0.20[c]	174.7±0.20[d]	P=0.001	P=0.001	P=0.001
	DHS	200.5±0.32[e]	198.7±0.15[f]	197.3±0.20[g]	192.5±0.20[h]			

†*Means of triplicate analyses ± standard deviation.* RM ANOVA, Repeated measured ANOVA with between-subject factor
[a]*Values within a rownot sharing the same superscript letter are significantly different ($P < 0.05$)*;NS,non significant
WS,whole seed; DHS,dehulled seed ;IVPD, In-vitro protein digestibilty

4.4.3 The effect of fermentation time on moisture, protein, fat and *in vitro* protein digestibility (IVPD) of lupin natto

The effect of fermentation time and source on lupin natto (*B. subtilis* natto fermented) were determined. Results are presented in table 5.3 and there was a significant effect of fermentation time, source of lupin and there was an interaction between fermentation time × source of protein content of lupin natto (Table 4.3). Protein content of 48 hours fermented lupin natto was the highest (41.65%). Also, lupin natto prepared from dehulled seeds had significantly higher ($P < 0.05$) protein content than whole seed lupin natto. Therefore, with respect to protein content, 48 h fermented lupin natto is nutritionally valuable.

Similarly, there was a significant effect of fermentation time, source and source x fermentation time on moisture content of lupin natto. 0 h fermented lupin natto had highest moisture ($P < 0.05$) content and 48 h fermented lupin natto had the lowest. Moisture content found in this study (53.4-59.4 for WS and 46.2-50.4 for DHS%) is comparable with acceptable maximum moisture content of natto (Wei et al., 2001). Tiara and others (1983) indicated that the average moisture content of natto as 58.1-60.6% and (Wei et al., 2001) reported the similar results.

Also, in line with moisture drop sample weight of lupin natto was decreased. Decreasing of sample weight of lupin natto was significantly ($P < 0.05$) affected by source and fermentation time. Lupin natto samples fermented for 12 h, 24 h and 48 h had significantly lower ($P < 0.05$) sample weights compared to 0 h fermented lupin natto.

Similar to tempeh, Initial weight of each sample was kept constant at 200 g. In pairwise comparison 0,12, 24, 48 h fermented samples each one was significantly different to others. The reduction of weight of the samples has a relationship with reducing moisture levels with fermentation time. The fat content of lupin natto was significantly affected ($P < 0.05$) by fermentation time and source of lupin where the 12 h sample had the highest amount. The 48 h fermented samples had lowest ($P < 0.05$) fat content. This was common to both whole seed and de-hulled seed lupin natto. However, de-hulled seed lupin natto had significant ($P < 0.05$) higher fat content compared to whole seed. As shown in Table 4.3 there was a significant effect of fermentation time on IVPD of lupin natto. However, there was no significant effect ($P < 0.05$) of source (WS & DHS) on IVPD of lupin natto. Also, source (WS & DHS) and source x fermentation time did not have significant effect on IVPD. Both WS and DHS lupin natto which was fermented up to 24 h had highest ($P<0.05$) IVPD. Therefore, 24 h fermentation is the optimum for natto preparation. Also, nutritionally important highest digestible lupin natto can be prepared from 24 h fermentation. IVPD of lupin natto was increased 0-24 h by 8.5% then it was decreased by 4.2% at 48 h. As reported by (Embaby, 2010), the IVPD values of raw sweet and bitter lupin seeds were 79.46% and 78.55% respectively. These IVPD values are comparable to the data reported in this study (Table 4.2 for lupin tempeh and Table 4.3 for lupin natto). Also, in another experiment, different legumes i.e. chickpea, lentil, white bean and pink motted cream reported to have similar to value of IVPDs. Further, it was reported that processing steps such as (soaking, soaking + cooking and soaking + cooking + dehydration) increased the IVPDs and values were 80-90.9% for chickpea, 85-95.9% for lentil, 79.1-90.0% for white bean and 79.6-91.3% for pink motted cream bean (Martín-

Cabrejas et al., 2009). Moreover, the effect of soaking, cooking and autoclaving on some ANFs and IVPDs of Vigna. Aconitifolia and V. sinensis have been studied and IVPDs were enhanced by 12.5 and 14.8% respectively (Embaby, 2010; Vijayakumari, Siddhuraju, Pugalenthi, & Janardhanan, 1998). As a result of heating, antinutrients factors in raw beans are destroyed, then enzymatic digestion is improved. (Fuller, 2012) This may have led to improve IVPD after above processing methods. Also, due to soaking, digestive enzymes are activated and some antinutrients get diluted and IVPD is increased.

Table 4.3. Australian sweet lupin natto; effect of fermentation time on moisture, fat, protein and *in vitro* protein digestibility (IVPD) †

Nutrient component & source	Fermentation time (h)					RM-ANOVA		
	Source	0	12	24	48	Time	Source	T x S
Moisture (%)	WS	59.4±0.29[a]	58.4±0.04[a]	57.1±0.05[a]	53.5±0.05[b]	P=0.002	P=0.001	P=0.003
	DHS	50.4±0.29[c]	49.1±0.04[d]	48.1±0.05[e]	46.2±0.05[f]			
Fat (%)	WS	5.8±0.28[a]	6.7±0.08[b]	6.5±0.04[c]	7.8±0.20[d]	P=0.001	P=0.005	P=0.002
	DHS	6.2±0.12[e]	6.9±0.04[f]	7.3±0.03[g]	8.8±0.19[h]			
Protein (%)	WS	27.2±0.22[a]	28.6±0.479[a]	29.2±0.47[b]	29.8±0.19[c]	P=0.001	P=0.001	P=0.004
	DHS	35.9±0.22[d]	37.1±0.47[d]	39.8±0.47[e]	41.7±0.19[f]			
Sample weight	WS	200.0±0.00[a]	190.2±1.06[b]	185.1±0.38[c]	165.7±0.17[d]	P=0.001	P=0.02	P=0.001
	DHS	200.0±0.00[e]	185.6±1.061[f]	178.0±0.38[g]	165.5±0.17i			
IVPD (%)	WS	83.2±1.003[a]	88.5±1.22[a]	93.0±0.972[b]	89.3±2.41[a]	NS	P=0.03	P0.05
	DHS	87.5±1.29[a]	88.4±1.22[a]	94.0±0.9b	90.2±2.41[a]			

† *Means of triplicate analyses ± standard deviation. RM ANOVA, Repeated measured ANOVA with between-subject factor*
[a]*Values within a rownot sharing the same superscript letter are significantly different (P < 0.05).*
RL, raw lupin; SL, soaked lupin; BL, boiled lupin; WS,whole seed; DHS,dehulled seed. *SL, soaked lupin; BL, boiled lupin;*

WS,whole seed; DHS,dehulled seed.

4.4.4 The effect of fermentation time on dietary fibre profile of lupin natto

Dietary fibre components, insoluble dietary fibres (IDF), soluble dietary fibre (SDF) and total dietary fibre (TDF) of raw lupin and lupin natto samples were measured and table 4.4 shows the available dietary profile data.

IDF content of raw lupin (RL), lupin natto (0 h fermented), and lupin natto (48 h fermented) are 44.32, 43.23 and 48.42 % for whole seed (WS) and 32.63, 32.45 and 35.54 for de-hulled seed (DHS) respectively. (Veena, Urooj, & Puttaraj, 1995) found that there is an increase in TDF and IDF of Bengal gram, cow pea and green gram during fermentation. TDF values of those legumes have been reported as 25.6, 23.2 and 24.5 (g 100 g^{-1} db) respectively. Raw lupin exhibited high amount of relevant level of 46.42% HMWDF(TDF) and higher than other similar legume data (Veena et al., 1995). The process of tempeh fermentation did not show significant ($P < 0.05$) decrease in IDF and HMWDF but natto fermentation showed significant ($P < 0.05$) increase of IDF and HMWDF which agrees with previous findings of (Martín-Cabrejas et al., 2004). There was a significant effect ($P < 0.05$) of product (natto and tempeh) on dietary fibre components of ASL (RL, LT, LN) i.e. lupin tempeh has significantly lower IDF than natto but there was no such difference in SDFP and HMWDF. The increase of IDF of natto may be due to lower accessible of microorganism to the insoluble fibre component during fermentation. Moreover, in mould fermentation, mycelium could have grown through and digested cell walls of seeds and this may have led to reduce IDF, but bacteria do not have such ability. With the decrease in the IDF there was a corresponding increase in SDF (SDFP) and this signifies that significant effect of fermentation time/period on soluble dietary fibre fraction (SDFP)

whereas 48 h fermented natto and tempeh had highest (P < 0.05) SDFP compared raw lupin and 0 h fermented samples. Also 15% reductions of IDF and HMWDF of both natto and tempeh might be due to the use of cellulose, the main component of the legume, by microorganism present in the fermentation medium. Cellulose as predominant fraction of lupin hull, can be regarded as a source of glucose during chemical and enzymatic microbial processing. Also, decayed component produced by microbial breakdown are of particular importance from nutritional and technological point of view. However contrary results have been reported by (Martín-Cabrejas et al., 2004) and it was justified that it may be due to incomplete recovery by ethanolic precipitation in the fibre analysis of SDFP. Also, there was a significant effect (P < 0.05) of source (WS and DHS) on dietary fibre of raw lupin and fermented counterparts. IDF and HMWDF of whole seed is significantly higher (P < 0.05) than IDF and HMWDF of dehulled seed (e.g IDF of Whole seed RL is 44.32% and IDF of Dehulled seed RL is 32.68 Table 4.3) However there was no significant (P < 0.05) difference in SDFP.As legumes contain higher amount of SDF compared to cereal and tuber food, they owe special functional character called "Lente effect" (slow releasing) In the present study, it was observed that the SDF (SDFP) content was higher in lupin natto and tempeh which was fermented for 48 h compared to raw lupin and 0 h fermented (the samples with added starter culture but not fermented well). In fact, it is reported that legumes such as Asian traditional fermented legume food such as miso, natto and tempeh consumption have been reported to have reduced chronic diseases. However, certain disagreement between earlier findings and the results obtained in this study can be explained by differences in methods used as well as species, climatic and soil conditions. Depending on the varieties the dietary fibre had different fractional

compositions. IDF content of raw lupin (RL), lupin natto (0 h fermented), and lupin natto (48 h fermented) are 44.32, 43.23 and 48.42% for whole seed (WS) and 32.63, 32.45 and 35.54 for de-hulled seed (DHS), respectively. (Veena et al., 1995) found that there is an increase in TDF and IDF of Bengal gram, cow pea and green gram during fermentation. TDF values of those legumes have been reported as 25.6, 23.2 and 24.5 (g 100 g^{-1} db.) respectively. Raw lupin exhibited high amount of relevant level of 46.42% HMWDF(TDF) and higher than other similar legume data (Veena et al., 1995). The process of tempeh fermentation did not show significant ($P < 0.05$) decrease in IDF and HMWDF but natto fermentation showed significant ($P < 0.05$) increase of IDF and HMWDF which agrees with previous findings of (Martín-Cabrejas et al., 2004). There was a significant effect ($P < 0.05$) of product (natto and tempeh) on dietary fibre components of ASL (RL, LT, LN) i.e. lupin tempeh has significantly lower IDF than natto but there was no such difference in SDFP and HMWDF. The increase of IDF of natto may be due to lower accessible of microorganism to the insoluble fibre component during fermentation. Moreover, in mould fermentation, mycelium could have grown through and digested cell walls of seeds and this may have led to reduce IDF, but bacteria do not have such ability. With the decrease in the IDF there was a corresponding increase in SDF (SDFP) and this signifies that significant effect of fermentation time/period on soluble dietary fibre fraction (SDFP) whereas 48 h fermented natto and tempeh had highest ($P < 0.05$) SDFP compared raw lupin and 0 h fermented samples. Also ~ 15% reductions of IDF and HMWDF of both natto and tempeh might be due to the use of cellulose, the main component of the legume, by microorganism present in the fermentation medium. Cellulose as predominant fraction of lupin hull, can be regarded as a source of glucose during chemical and enzymatic

microbial processing. Also, decayed component produced by microbial breakdown are of particular importance from nutritional and technological point of view. However contrary results have been reported by (Martín-Cabrejas et al., 2004) and it was justified that it may be due to incomplete recovery by ethanolic precipitation in the fibre analysis of SDFP. Also, there was a significant effect ($P < 0.05$) of source (WS and DHS) on dietary fibre of raw lupin and fermented counterparts. IDF and HMWDF of whole seed is significantly higher ($P < 0.05$) than IDF and HMWDF of dehulled seed (e.g IDF of Whole seed RL is 44.32% and IDF of Dehulled seed RL is 32.68% (Table 4.3). However, there was no significant ($P > 0.05$) difference in SDFP. As legumes contain higher amount of SDF compared to cereal and tuber food, they owe special functional character called "Lente effect" (slow releasing) In the present study, it was observed that the SDF (SDFP) content was higher in lupin natto and tempeh which was fermented for 48 h compared to raw lupin and 0 h fermented (the samples with added starter culture but not fermented well). In fact, it is reported that legumes such as Asian traditional fermented legume food such as miso, natto and tempeh consumption have been reported to have reduced chronic diseases. However, certain disagreement between earlier findings and the results obtained in this study can be explained by differences in methods used as well as species, climatic and soil conditions. Depending on the varieties the dietary fibre had different fractional compositions.

In summary, there was no significant difference (*P* < 0.05) between natto and tempeh on IDF, SDFP and HMWDF. However, whole seed had ~ 70% more IDF and HMWDF than de-hulled samples but there was no difference in SDFP. Fermentation time remarkably increased (*P* < 0.05) SDFP in both natto and tempeh (~ 50%) while giving ~ 15% reduction of IDF and HMWDF. Although lactic fermentation and natural fermentation have been carried out and reported commonly on the effect of dietary fibre components, the alkaline fermentation by mould specially *R. oligosporus* and bacteria *Bacillus subtilis* has not been well documented on legumes especially on lupin. Finally, different processing approaches including fermentation improves Australian sweet lupin's nutritional qualities including protein digestibility, fibre profile three by lupin seed may be advocated as a high nutritious and functional food for human. Also, as it is low cost legume, it will be better choice for using as a valuable protein food source for developing countries.

Table 4.4. Australian sweet lupin natto; effect of fermentation time on dietary fibre profile (IDF, SDF and TDF) †
(mg 100 g^{-1} db)

Dietary fibre component		Fermentation status			RM-ANOVA		
		RL	0 h	48 h	Time	Source	T x S
IDF	WS	44.3±0.23[a1]	48.4±0.13[c2]	45.9±0.42[d4]	P=0.001	P=0.001	P=0.001
	DHS	32.6±0.05[a1]	35.5±0.00[c2]	10.9±0.64[d4]			
SDF	WS	2.3±0.33[a1]	2.1±0.31[a2]	4.3±1.77[b4]	P=0.035	NS	NS
	DHS	1.2±0.13[a1]	2.74±1.36[c2]	4.9±0.18[d4]			
TDF	WS	46.4±1.42[a1]	49.9±0.50[a2]	49.0±0.78[b4]	P=0.001	P=0.002	P=0.001
	DHS	34.5±1.91[a1]	39.3±0.37[c2]	16.3±0.42[d4]			

† *Means of triplicate analyses ± standard deviation;* RM ANOVA, Repeated measured ANOVA with between-subject factor ; NS, non significant

[a] *Values within a rownot sharing the same superscript letter are significantly different (P<0.05)*

RL, raw lupin; WS,whole seed; DHS,dehulled seed;IDF,Insoulbe dietary fibre; SDF, Soluble dietary fibre; TDF, Total dietary fibre

Table 4.5. Australian sweet lupin tempeh; effect of fermentation time on dietary fibre profile (IDF, SDF and TDF) † (mg 100 g^{-1} db.)

Dietary fibre component		Fermentation status			RM-ANOVA		
		RL	0 h	48 h	Time	Source	T x S
IDF	WS	44.3+0.23^{a1}	43.2+3.88^{a1}	37.8+0.23^{b3}	P=0.012	P=0.001	NS
	DHS	32.6+0.05^{a1}	31.6+4.02^{a1}	18.8+2.45^{b3}			
SDF	WS	2.3+0.33^{a1}	1.2+0.23^{a1}	5.5+0.68^{b3}	P=0.001	NS	P=0.046
	DHS	1.2+0.13^{a1}	1.5+0.08^{a1}	6.5+0.52^{b3}			
TDF	WS	46.4+1.42^{a1}	45.7+7.77^{a1}	39.7 +1.92^{b3}	NS	P=0.014	NS
	DHS	34.5±1.91^{a1}	32.4±2.12^{a1}	37.8±2.72^{b3}			

† *Means of duplicate analyses ± standard deviation;* RM ANOVA, Repeated measured ANOVA with between-subject factor; NS, non significant

[a] *Values within a row not sharing the same superscript letter are significantly different ($P<0.05$).*

RL, raw lupin; WS,whole seed; DHS,dehulled seed;IDF,Insoulbe dietary fibre; SDF, Soluble dietary fibre; TDF, Total dietary fibre

4.5 Conclusion

This is the first report on IVPD, dietary fibre profile of lupin natto and tempeh. Although, some nutritional composition data of lupin tempeh was available earlier, all the nutritional composition data including fibre profile of lupin natto is novel data. IVPD and fibre values and are affected by soaking, dehulling and boiling specifically, IVPD was increased by them. Furthermore, nutritional components such as protein, fat and dietary fibres and IVPD are increased by fermentation, indicating that *R. oligosporus* and *B. subtilis* fermentation of lupin result improvement in the nutritional value of lupin.

CHAPTER 5

EFFECT OF DIFFERENT FERMENTATION APPROACHES ON GENISTEIN AND DAIDZEIN CONTENT OF AUSTRALIAN SWEET LUPIN (*LUPINUS ANGUSTIFOLIUS*)

5.0 Connecting statement

Findings of previous chapter concluded that the fermentation increased nutritional value of ASL. This chapter will be discussing the effect of fermentation on some bioactive compound such as daidzein and genistein.

5.1 Introduction

Fermentation of legume seeds has been extensively used as bio-process to improve its nutritive qualities and thereby use them as functional food (Fernandez-Orozco et al., 2007). During fermentation, some of the seed reserves are digested by microorganisms (bacteria, fungi and mould) for their own metabolism. In return, these microorganisms enrich the medium with their metabolites (Deshpande, 2000). Further, fermentation increases the level of free amino acid, available carbohydrate, dietary fibre and bioactive compounds (Shieh, Beuchat, Worthington & Phillips, 1982; Salunkhe, Kadam, 1989; Refstie, Sahlström, Bråthen, Baeverfjord, & Krogedal, 2005). Natto, miso, tempeh and tofu are some indigenous Asian soya fermented foods which originated in Japan, Indonesia and China respectively, but are now being consumed all over the world (Salunkhe & Kadam, 1989). Importantly these soybeans based fermented foods are rich in phytoestrogens genistein and daidzein which exist as 7 O-glycosides

(Sirtori, Arnoldi, & Johnson, 2005). Phytoestrogens are plant derived isoflavone compounds which has similarity in structure and function to human estrogen (Dixon, 2004). There is ongoing evidence for beneficial effects of phytoestrogens on glucose metabolism, lipid metabolism, osteoporosis and health issues related to menopause (Sirtori, Agradi, Conti, Mantero, & Gatti, 1977; Fujimoto et al., 1991; Anderson , Johnstone , & Cook-Newell 1995; de Kleijn, van der Schouw, Wilson, Grobbee, & Jacques, 2002; Yang et al., 2010; Saha, Sadhukhan, & C. Sil, 2014). Treatment doses ranging from 0-2.5 mg kg^{-1} 17β–estradol or genistein in rats resulted in protection against cell proliferation. Some epidemiological studies have suggested that Asian women have lower rate of breast cancer than western women. To explain above epidemiologic observation estrogen mimicking effect of phytoestrogen was suggested as a possible mechanism (Shimokado, Yokota, Umezawa, Sasaguri, & Ogata, 1994; Kentaro Shimokado, Umezawa, & Ogata, 1995). However, these health benefits of soya and phytoestrogens is a controversial issue as number of research findings have indicated inverse results as well (Maxwell Parkin, 1989; Anderson et al., 1995; Yuan, Wang, Ross, Henderson, & Yu, 1995; Nestel et al., 1997; Greaves, Parks, Williams, & Wagner, 1999). Due to these negative findings health authorities of some countries have indicated maximum daily intake level for phytoestrogen (Sirtori et al., 2005). According to recent findings, serum genistein level >10 μM is associated with inhibition of tumour growth whereas serum genistein level < 10μM is associated with tumour growth. However, attaining the target >10 μM serum genistein level is doubtful due to the bioavailability and other factors such as dietary habits. Lupin is a legume with high protein but low in phytoestrogens (geistein, daidzein). Therefore, preparation of indigenous Asian fermented legume foods such as

natto and tempeh using other comparable legume is an interesting alternative to avoid potential overdosing of phytoestrogens.

Lupin is a similar legume to soya in some of its food characteristic though it has lower amount of isoflavone, phytoestrogen especially daidzein and genistein (Sirtori et al., 2004). However, fermentation (Bavia et al., 2012; Haron, Ismail, Azlan, Shahar, & Peng, 2009) and germination (Dueñas, Hernández, Estrella, & Fernández, 2009; Suparmo & Markakis, 1987) are processing techniques in which the hydrolysis of glycosides can occur as a result of digestion activities forming aglycones (Ferreira et al., 2011; Wu & Chou, 2009). Fermentation converts glycosylated isoflavone (genistin and daidzin) into their aglycone counterpart (genistein and daidzein) which eventually increase their bioavailability when ingested by humans (Haron et al., 2009; Izumi et al., 2000). Black soybean fermented *B. subtilis natto* showed significant decrease of genistin and daidzin content while it increased genistein and daidzein content (Hu et al., 2010). According to findings of many research around the world, lupin contains high (116-274 mg 100 g^{-1}), moderately high (57-118 mg 100 g^{-1}) or low (13.6-16.6 mg 100 g^{-1}) amount of daidzein and genistein depending on the species, cultivars and or even geographical areas (Wang & Murphy, 1994; Genovese, Hassimotto, & Lajolo, 2005; Ranilla, Genovese, & Lajolo, 2009). However, (Ranilla et al., 2009) reported that, there is negligible amount of daidzein and genistein in *L. angustifolius* in Brazilian variety. Therefore, it is worth to study the daidzein and genistein content of ASL.

Natto is a Japanese traditional fermented soya been food produced from *B. subtilis natto*, is known as functional food due to its anti-coagulant effect and blood pressure suppressing effect (Hsu, Lee, Wang, Lee, & Chen, 2008; Taniguchi-Fukatsu et al., 2012). Tempeh is a traditional Indonesian soya

fermented food with many health benefits obtained by fermentation with *R. oligosporus* (Karyadi & Widjaja, 1996). Due to its cost effectiveness and comparable nutritional profile, lupin has substitute to soya when producing some Asian indigenous fermented food (Fudiyansyah, Petterson, Bell, & Fairbrother, 1995). The aim of this study was to determine the phytoestrogen content of raw Australia Sweet Lupin (ASL), and to determine the effect of fermentation time, and source (Whole seed /Dehulled seed) on their content in lupin natto and lupin tempeh.

5.2 Materials and Methods

5.2.1 Raw materials and microbial culture used

Acetonitrile (HPLC grade), genistein, daidzein, dimethyl sulfoxide (DMSO), polytetrafluoroethylene microfilter (PTFE 0.20 mm pore size I.D.; 25 mm), ethanol, acetone reagent grade, acetic acid solution, tris base solution, hydro chloric acid and sodium hydroxide, were purchased from Sigma Aldrich, New South Wales, Australia. Millipore water was used for all mobile phases.

Lupin whole seeds (*L. angustifolius*) and de-hulled seeds were obtained from CBH group, Perth, Western Australia. *Bacillus subtilis* starter culture (ATCC 6633™ 10,000 CFU vitroids) was purchased from Sigma-Aldrich Australia and used as inoculum for producing natto. Commercially available tempeh starter from Cultures for Health, USA was used as *R. oligosporus* stater culture for making tempeh.

5.2.2 Methods

5.2.2.1 Preparation of lupin tempeh

Lupin tempeh was prepared as described in 3,3.2.6

5.2.2.2 Preparation of natto

Lupin natto was prepared as described in 3.3.2.5

5.2.2.3 Extraction, spiking and determination of genistein and daidzein content

Extraction of genistein and daidzein were carried out based on the method by (Greenfield, & Mulholland, 2000; Haron et al., 2009; Hutabarat,). About 1 g of sample was added to 40 mL of 96% ethanol and 10 mL of 2M hydrochloric acid. This was spiked with various volumes of stock solution containing 20% of standard daidzein and genistein. Then sample mixture was treated in a sonicator for 20 min followed by reflux for 4 h at 103 °C. The mixture was then made up to 50 mL with ethanol 96% and adjusted to pH 4 adding sodium hydroxide. Resulted samples were centrifuged (Beckman J2, PALO, ALTO, California, USA) at 800 g for 20 min. Finally, clear supernatant was passed through a Whatman filter paper (No.4) and 0.2 m polytetrafluro-ethylene micro filter (PTFE) to inject into a reverse phase high performance liquid chromatography (HPLC) (Agilent Technologies, California, USA).

Preparation of standard solutions

Standard solutions of genistein and daidzein were prepared according to method by (Haron et al., 2009). Each genistein and daidzein 10 mg pre-weighed samples were ordered from Sigma Aldrich and dissolved in 20 µL dimethylsulphoxide (DMSO) followed by addition of 96% ethanol to make 1M solution (Stock

solution). Then working standard solutions series 10-150 µM were prepared using mobile phase (acetonitrile (A) -water (B) 33:67, v/v) at a flow rate of 0.80 mL min^{-1} (Hutabarat et al., 2000). The column was C_{18} Nova-Pak (150 x 3.9 x mm I.D., 4µm) (stationary phase). The wavelength ranged from 200 to 400 nm and maximum absorption of daidzein is 249.8. The maxima for genistein was 259.2 nm.

Purity of standard

In order to test the purity of genistein and daidzein, they dissolved separately first in 20 µl DMSO followed by the addition of 96% ethanol. The purity of the standards was checked by injecting the stock solution into the HPLC and percentage purity was calculated by dividing the peak area of the compound by all peak areas and multiplying by 100.

5.3 Statistical analysis

Data were expressed as mean ± standard deviation of duplicate measurements. Data were analysed using, SPSS version 24.0 for windows (SPSS Inc, Chicago, USA). Repeated measures ANOVA with between group factors was used to determine the change and difference in daidzein and genistein content of Natto and Tempeh separately. Level of significance was set at $P < 0.05$.

5.4 Results and Discussion

Quantification of daidzein (Da) and genistein (Ge) in this experiment was based on validated rapid isocratic HPLC technique by (Hutabarat et al., 2000). In this study standard of Da and Ge eluted at 4.4 min and 8.8 min respectively. As shown in Figure 5.1 they were eluted at 4.6 and 8.8 in lupin samples.

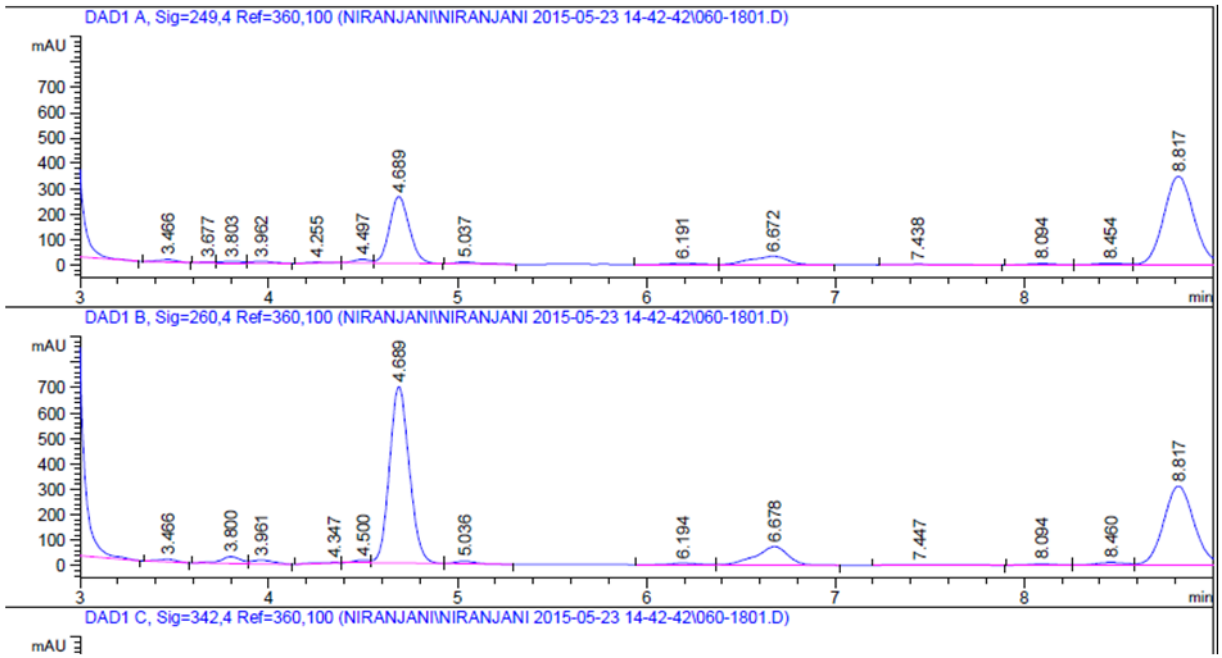
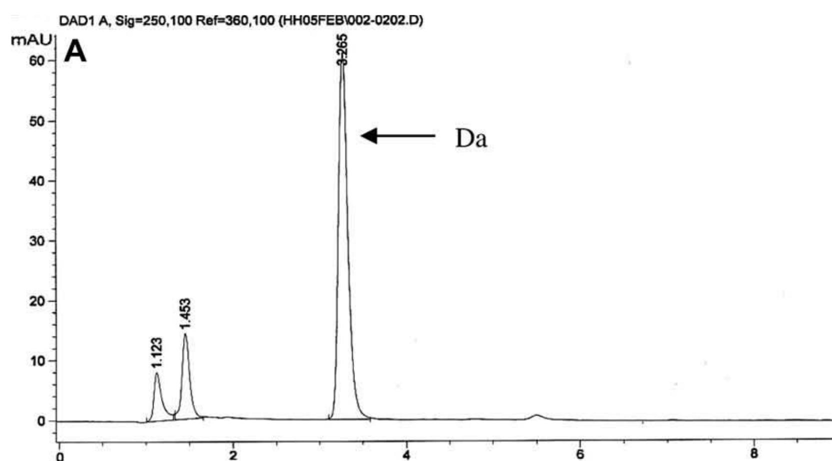
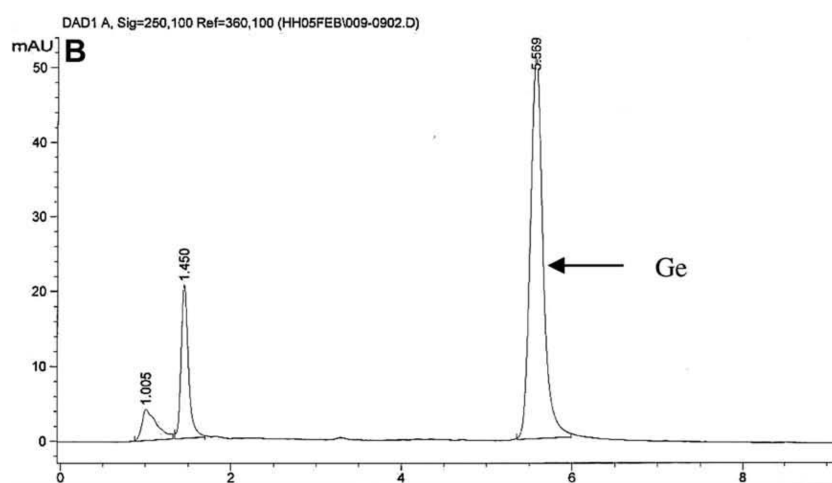


Figure 5.1. HPLC chromatograms of daidzein peak eluted at 4.6 min; genistein peak eluted at 8.8 min in sample





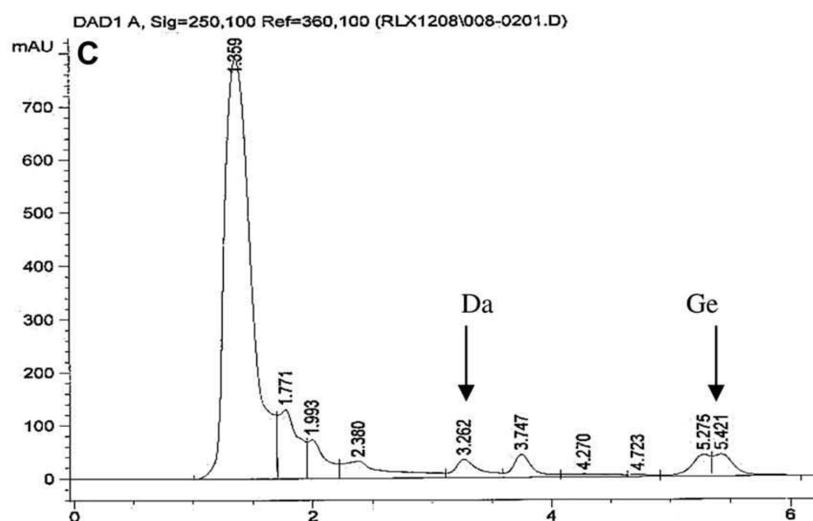


Figure 5.2 HPLC chromatograms of (A) daidzein standard peak eluted at 3.2 min; (B) genistein standard peak eluted at 5.5 min; and (C) daidzein and genistein peak eluted at 3.3
Source: Haron et al. (2009)

5.4.1 Effect of processing on genistein and daidzein content of Australian sweet lupin

Table 5.1 Genistein and daidzein content (mg/100g db) a of Australian sweet lupin (Lupinus angustifolius) as affected by different processing types

Isoflavone	Samples	RL^{b}	SL^{b}	BL^{b}
Ge	WS	0.49±0.01^{c}	0.42±0.01^{c}	0.13±0.01^{c}
	DHS	0.27±0.01^{d}	0.15±0.03^{d}	0.03±0.02^{d}
Da	WS	0.70±0.07^{e}	0.54±0.04^{e}	0.12±0.01^{e}
	DHS	0.59±0.09^{f}	0.41±0.02^{f}	0.11±0.01^{e}

RL, raw lupin; SL, soaked lupin (16 h); BL, boiled lupin (60 min); Ge, genistein; Da, daidzein; WS, whole seed; DHS, dehulled seed.
a Means of analyses ± standard deviation.
b Values within a column with the same superscript letter are not significantly different (P<0.05).

Table 5.1 gives the daidzein and genistein content (mg 100 g^{-1} db) of raw, soaked, and cooked (boiled for 1 h) lupin samples. The values were comparable to that previously reported Da 0.10 mg 100 g^{-1} db. and Ge 0.15 mg 100 g^{-1}db. (Bhagwat, 2008). There was a significant effect ($P < 0.05$) of processing types (raw, soaked and boiled) but there was no significant effect ($P < 0.05$) between sources whole seed and dehulled seed. However, in pair wise comparison all processing types raw, soaked and boiled showed significant differences ($P < 0.05$) among them. The decrease in daidzein from raw to boiled is half as much in lupin tempeh. However roughly the change from boiled to peak (around 0.6-0.7) is about the same in lupin tempeh (around 0.5-0.6). Processing methods soaking and cooking (boiling) has decreased daidzein and genistein content significantly ($P < 0.05$).

Figures 5.2-5.5 show De and Ge content of lupin natto and tempeh as affected by fermentation time (0-72 h) type of lupin (WS and DHS). Fermentation time, type of lupin (WS; DHS) and product (natto and tempeh) have shown significant effect ($P < 0.05$) on Da and Ge content of ASL.

5.4.2 Effect of Fermentation on daidzein content of Australian sweet lupin

As shown in Figure 5.2 and 5.3, Da content at 0 h is significantly difference (P < 0.05) from all the time points up to 72 h. Da content at 24 h is significantly differs (p<0.05) from all time point except for 36 h and 72 h. Da content at 36 h shows insignificant difference (P < 0.05) among time points 24, 48 and 72 h however it is significantly differs (P < 0.05) from 0 and 12 h. 48 h is significantly difference

(P < 0.05) from all the time points except for 36 h. 72 h is significantly difference from (P < 0.05) 0, 12 and 48 h whereas 24 h and 36 h are not.

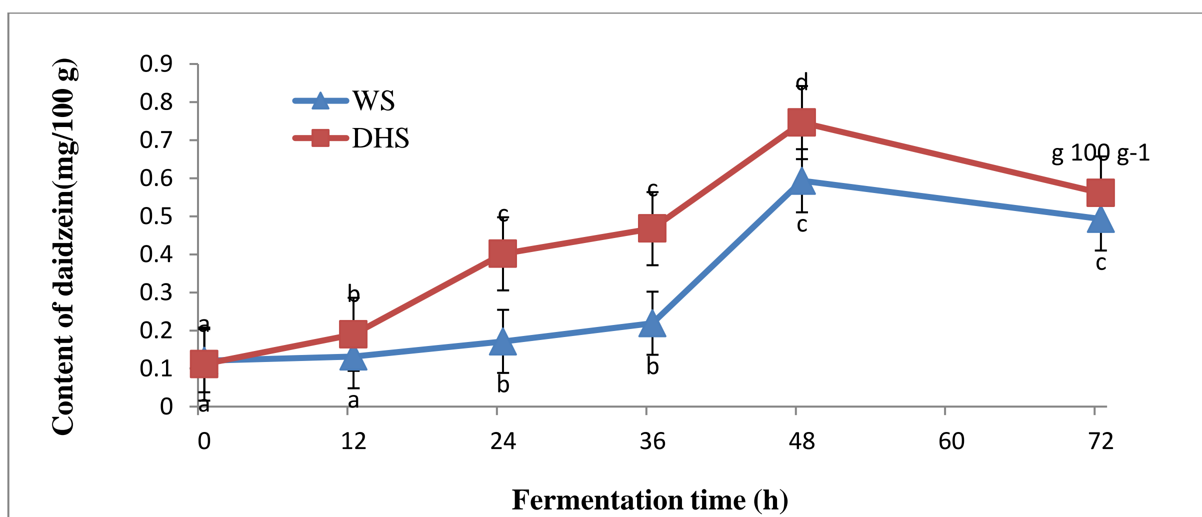


Figure 5.3. The change of daidzein content of tempeh analogue over 72 h of *R. oligosporus* assisted fermentation

WS, whole seed; DHS, dehulled seed
Mean N= 2, ± SEM

a-d denotes significantly difference values

Both WSLN and DHLN showed dramatic increase of daidzein during fermentation and in DHLN drastic change happened at 12-24 h while in WSLN it was 24-36 h. Both reached their peak value at 36h and then decreased up to 72 h. Maximum daidzein content can be seen at 36 h in both whole seeds and de-hulled, so this maybe optimum time point of fermentation of natto. Though fermentation time increased daidzein content of both WSLT and DHLT significantly ($P < 0.05$) the rate of increment is low compared to available content in raw lupin. Also, peak level was at 48 h for both sources whole seed and dehulled seeds.

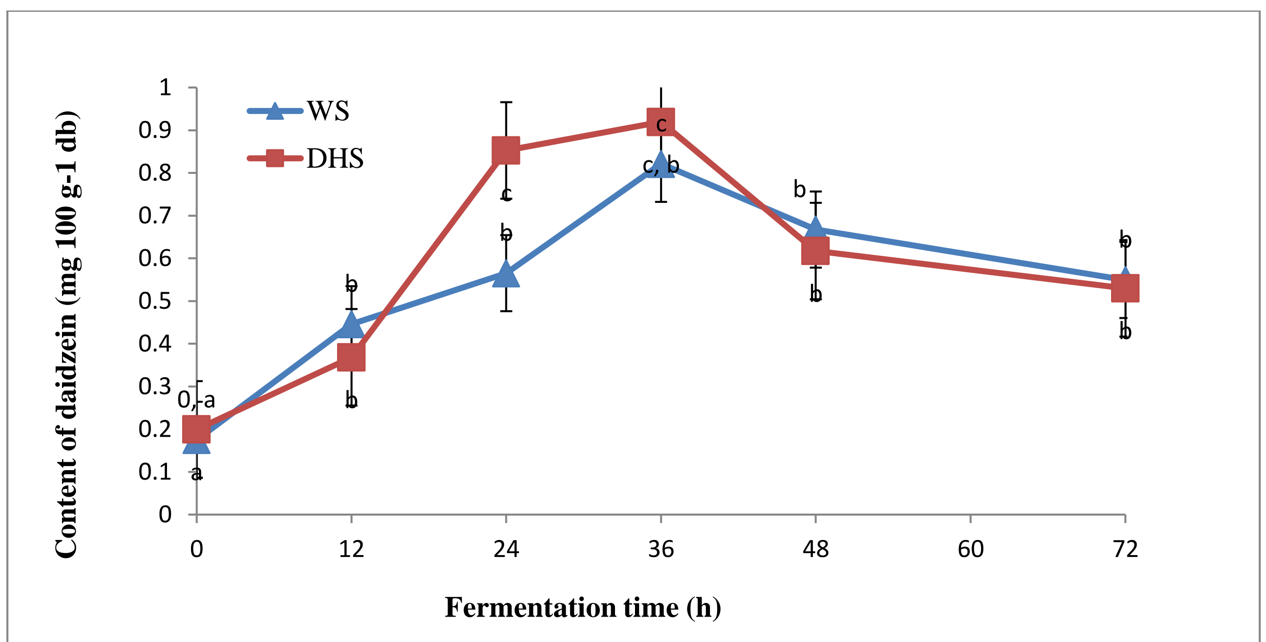


Figure 5.4. The change of daidzein content of natto analogue over 72 h of *B. subtilis* assisted fermentation to produce a natto analogue

WS, whole seed; DHS, dehulled seed
Mean ± SEM
a-d denotes significantly difference values

It can be observed that, fermentation time, time x source (WS/DHS), time x product (natto/tempeh) and time x source x product had significant effect on daidzein content of ASL.

5.4.3 Effect of Fermentation on genistein content of Australian sweet lupin

Figure 5.4-5.5 presents the change of genistein content of “lupin natto” the product obtained from lupin fermentation with (bacteria) *Bacillus subtilis* and “lupin natto” the product which is obtained from lupin fermentation with (mould) *Rhizopus oligosporus* respectively.

As shown in Figure 5.4, WSLN had a significant increase of genistein content after 12 h of fermentation. It reached highest value of (2.38 mg 100 g^{-1} db.) at 36 h which is the optimum fermentation time for the production of natto. Similarly, in DHLN, there was a significant increase of genistein content ($P < 0.05$) at 12 h after fermentation and it was peaked at 36 h. However, rate of increase of genistein of WSLN is greater than DHLN.

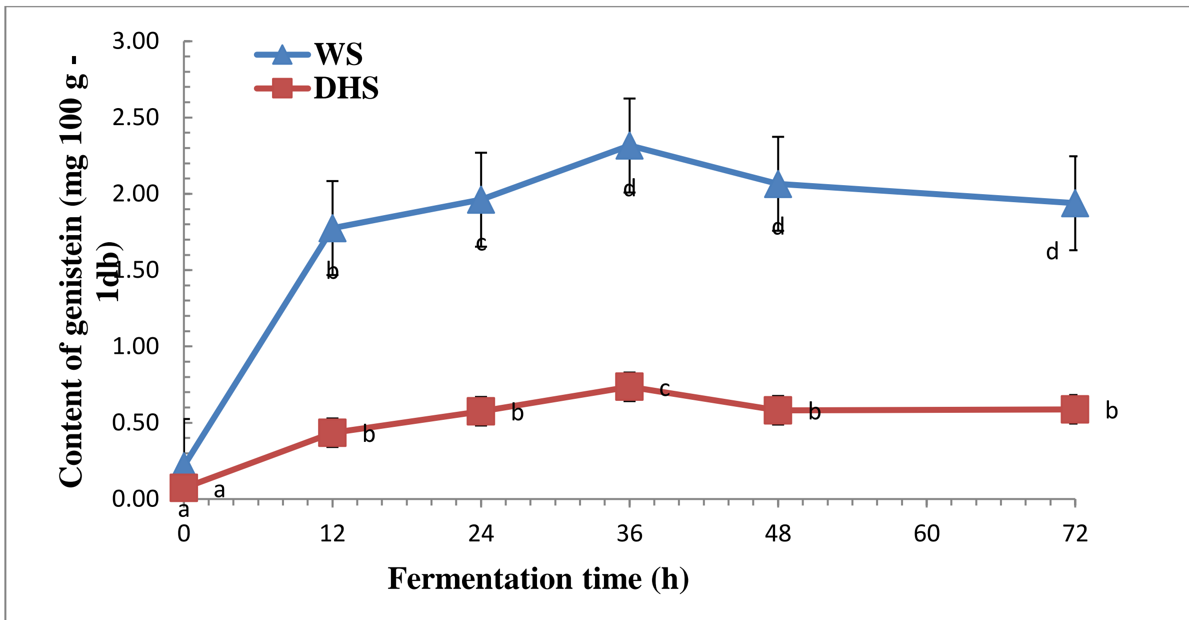


Figure 5.5. The change of genistein content of natto analogue over 72 h of *Bacillus subtilis* assisted fermentation

WS, whole seed; DHS, dehulled seed

Mean ± SEM

a-d denotes significantly difference values

During fermentation WSLT showed dramatic increase between 24-48 h and it was peaked at 48 h and then got platued. Rate of increment of genistein of DHLT was comparatively low, but again was peaked at 48 h and then platued similarly. Therefore 48 h could be considered as optimum time for tempeh production with regards concentration of these compounds.

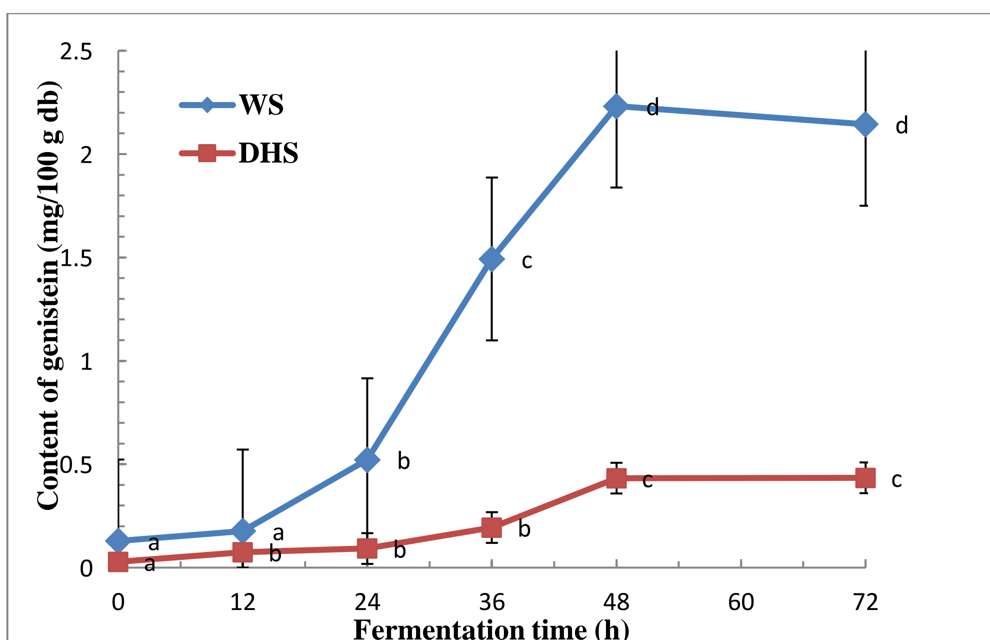


Figure 5.6 The change of genistein content of tempeh analogue over 72 h of *R. oligosporus* assisted fermentation

WS, whole seed; DHS, dehulled seed
Mean ± SEM
a-d denotes significantly difference values

5.5 Discussion

As shown in Table 5.1 and Figure 5.2 - 5.5 whole seed raw lupin (WSRL) contains highest amount of Da (0.7 mg 100 g^{-1}) and whole seed lupin natto (WSLN) 36 h contains highest content of genistein (2.32 mg 100 g^{-1}) compared to amount in soybean (60 mg 100 g^{-1}) (Bhagwat, 2008). However with reference to USDA online data as shown in Table 1 lupin contains 0.1 mg 100 g^{-1} of Da and 0.15 mg 100 g^{-1} Ge. These values are extremely lower when compared to soybean values (Soy seed Australia 62 mg 100 g^{-1}, soy natto 33.22 mg 100 g^{-1}, soya tempeh 22.66 mg 100 g^{-1}). Sirtori, 2004 reported that *Lupinus albus* also contains low amount of daidzein and genistein (0.56 mg 100 g^{-1}) which is very much agrees with our present study findings (Sirtori et al., 2004). Further Our

findings agrees with Ferreira (Ferreira et al., 2011) whom showed that reducing of glycosides and increasing of aglycones (Da and Ge) during fermentation stage and other processing types. Moreover the amount of aglycone genisetin and daidzein are affected by the temperature of the medium and temperature was kept as constant factor in the present study (Wu & Chou, 2009).

Also, according to present results daidzein content of WS and DHS in both lupin natto and tempeh are almost similar, and which explains that they are mostly distributed in cotyledons/kernels. In contrast genistein content is always higher in WS than DHS. Therefore, it can be concluded that genistein is distributed in both seed coat and cotyledon or mostly in the hull. Moreover, during fermentation, genistein in whole seed is increased remarkably. This is due to conversion of seed coat genistin (glycosylated form) to genistein (aglycone form) by action of microorganism (Kuo, Wu, & Lee, 2012).

5.6 Conclusion

Lupin seeds contain very low amount of phytoestrogen isoflavone compounds Da and Ge compared to very high reference value of soybean. Processing methods soaking, and cooking reduces the amount of Da and Ge due to leaching with water and heat destruction. However, fermentation is a bio process to increase the amount of isoflavone (Da by 700% and still effect of fermentation has not become a reason for overdosing of phytoestrogens in fermented lupin. Therefore, developed functional food ingredient from fermented lupin is safe to use in concern to phytoestrogen controversial issue.

5.7 Limitations

Also, as a reference value, USDA online data of soybean was used instead; soybean control had been used for this experiment. Further diminishing of other isoflavone such as glycosides (daidzin and genistin) has not been determined. These compounds were not detected due to limited resources and time/out of scope.

CHAPTER 6

THE EFFECT OF DIFFERENT FERMENTATION APPROACHES ON MINERAL AND TRACE ELEMENT COMPOSITION AND BIOAVAILABILITY OF AUSTRALIAN SWEET LUPIN (*LUPINUS ANGUSTIFOLIUS*)

6.1 Connecting Statement

After successful development of novel natto and tempeh analogues from lupin (chapter 3), significant improvements in nutritional composition (chapter 4), daidzein and genistein content and their interaction during fermentation (chapter 5) have been reported. In this chapter, the focus will be on mineral composition and effect of fermentation on mineral bioavailability of the Australian sweet lupin (ASL), whole seed (WS) and dehulled seed (DHS) natto and lupin tempeh.

6.2 Introduction

Legumes are commonly advocated in all diets around the world because of their beneficial nutritional effects, functional food activity and health benefits over chronic diseases and inexpensive alternative to animal protein (Kouris-Blazos, 2016). Moreover legume seeds are valuable source of minerals and trace elements. However bioavailability of minerals is diminished due to existence of anti-nutritional factors, such as saponins, polyphenols and phytate. Effective removal of the above anti-nutritional factors can be acquired by applying different food processing techniques such as soaking

in water, cooking (boiling, steaming and autoclaving) and some bio processes such as germination and fermentation (Xu & Chang, 2008). During these processes either naturally available enzymes are activated or new enzymes are generated by microorganisms. Many different kinds of legumes are commonly consumed and the lupin is gaining increased importance among them as it has great potential for human nutrition (Jayasena, Leung and Nasar- Abbas , 2010; van Barneveld, 2007; Villarino et al., 2016). The demand for lupin seed preparations and lupin flour based food products is increasing due to their evidence based health benefits (Arnoldi, Boschin, Zanoni, & Lammi, 2015; Rumiyati, 2010). Nutritional composition of lupin is unique and it is a valuable source protein, dietary fibre, vitamins, and nutritionally essential minerals for human nutriion (van Barneveld, 2007). On the other hand, lupin does contains some anti nuttritional factors (ANFs) such as tripsin inhibitors and phytates. It also contains varying levels of phenolic compounds, such as catechins and rutin. These compounds are also considerd as factors with negative effects as they interfere with absorption, especially minerals. Furthermore, lupin is rich in minerals and trace elements and (Hung, Handson, Amenta, Kyle, & Yu, 1988) reported that most of the minerals are present at a much higher concentration in the endosperm than the hull with the exception to the calcium (Ca) and aluminium (Al). However, lupin contains low level of Ca and phosphorus (P) but are similar or even levels of trace elements such as iron (Fe), zinc (Zn) and copper (Cu) when compared to other legumes. Another striking observation is the very high level of manganse (Mn) found in some varieties of *L. albus* (350 mg 100 g^{-1}) in comparison to *L. angustifolius* (4 mg 100 g^{-1}) (Hung et al., 1988). Mineral absorption from legumes is often very low although they are rich in various important minerals and trace elements (Sandberg & Svanberg, 1991).

This poor absorption is attributed to their content of bran in which dietary fibre components can bind minerals and high amount of phytates, polyphenols, oxalates and other antinutritional factors (ANFs) reducing mineral absorption. Even mineral-mineral interaction has inhibitory action on absorption of minerals and trace elements (Fairweather & Hurrel,1996). Fermentation however is a known processes for removal of these ANFs. As reported by (Sandberg, 1991) iron solubility of sorghum was increased after fermentation. The bioavailability of a mineral is defined as the fraction of ingested component that is absorbed and available for utilization of the body physiological function. The most accurate way to determinine bioavailability are *in vivo* studies. However, another promising method is *in vitro* experiments, as they are inexpensive and less time consuming (Ricardo, 1983). Morris & Ellis (1985) showed that absorption of Ca plants foods was reduced by phytic acid. Heany et al., (1995) found that, Ca uptake of high phytate soybean was 31% compared to lower phytate soybean (41%). Considering mineral -mineral interaction, Cr has shown inhibitory effects on bioavialability of Zn, Fe, and Ca. It is believed that Cr and Zn are absorbed by similar mechanism that leads to this inhibition (Seaborn & stoecker,1990). *In vitro* Cu forms complexes with phytates, hemicelluloses and lignin (Cheryan, 1980) and thus there is an inhibitory effect of dietary fibre on Cu absorption. This effect can however be reduced by α-cellulase and phytase enzyme activity during legume fermentation (Turland, 1995). The addition of Ca (165 mg) to a hamburger meal was shown to reduce the haem iron absorption (Hallberg et al., 1992) again demonstrating mineral-mineral interaction. Phyti acid is found in legume seeds and is a major determinant of the low iron bioavailability in these foods. It is thought to form an insoluble complex with Fe, and other mineral and peptide degradation products

in the intestinal lumen, from which the Fe cannot be absorbed (Hurrel et al., 1920). Phytic acid is a major Fe absorption inhibitory factor in isolated soy protein. Fe absorption is increased significantly when phytic acid free soy protein isolates were fed to adult in a liquid formulae meal (Hurrel et al.,1992) or to infants in infant formulae (Dardon et al.,1994). Some traditional food processing methods including fermentation can activate native phytases in grains which then degrade phytic acid and improve Fe absorption. Moeljopawiro et al. (1988) reported that Zn bioavailability was improved by *R. oligosporus* fermentation. Lactic acid fermentation of lupin increased Fe, Cu and Zn bioavailability, further, mineral availability of dehulled lupin was higher than in whole seed (Suliburska et al., 2009). However, the effect of fermentation by *R. oligosporus* and *B.subtilis* on mineral bioavailability of lupin has never been reported in depth.

Therefore, the aim of this study was to determine the total mineral content and mineral bioavailability in ASL WS and DHS when fermented to produce lupin natto and tempeh anaolgues.

6.3 Materials and Methods

6.3.1 Materials

The samples used in this experiment were: whole seed raw lupin (WSRL), dehulled raw lupin (DHRL), whole seed-soaked lupin (WSSL), dehulled soaked lupin (DHSL), whole seed lupin tempeh (WSLT), dehulled lupin tempeh (DHLT), whole seed lupin natto (WSLN), dehulled lupin natto (DHLN). Samples were prepared in triplicate as previously described in chapter 3 sections 3.2.3 and 3.3.3. Prior to analysis the raw lupin (WS and DHS) samples were milled into fine particles (100 micron) and the soaked lupin and tempeh/natto were freeze-dried and then milled.

All glassware was washed and rinsed in deionised water, soaked overnight in 10M nitric acid and rinsed again with deionised water. Disposable plastic tubes (mineral free) which were used for dialysis was received from National Measurement Institute (Perth, Western Australia).

6.3.2 Methods

6.3.2.1 Sample preparation and *in vitro* dialysis

The dialysis method as described by (Luten et al., 1996) was used to estimate the in vitro mineral availability of the dried/milled lupin, tempeh and natto samples. Duplicate 1 g samples were mixed with 5 mL distilled water, and the pH adjusted to 2 with 6M HCl. For simulated gastric digestion, 0.15 mL of freshly prepared pepsin solution (4 g pepsin, 2,500 units mg^{-1}, from Toxfree (Bedford St, Gillman SA) dissolved in 25 mL 0.1 M HCl was

added to each sample and the mixture was incubated at 37 °C for 2 h in a shaking water bath. Into one of the duplicate sample of the gastric digest freshly prepared pancreatine-bile extract mixture (400 mg pancreatine from Toxfree (Gillman, SA, Australia) and 2.5 g bovine bile extract from Sigma (St. Louis, MO, USA) in 100 mL 0.1 M $NaHCO_3$) was added, and the pH adjusted to 7.5 with 0.2M NaOH to determine the titratable acidity of the sample. Titratable acidity was defined as the amount of 0.2 M NaOH required to reach the pH 7.5. The second duplicate of the gastric digest was used for the simulated intestinal stage. In this stage, segments of dialysis bags with a molecular mass cut off at 10 kDa (Thermo Scientific, IL, USA) containing 25 mL of sodium bicarbonate (equivalent in moles to the NaOH used to determine the titratable acidity), were suspended in gastric digest in 50 mL centrifuge tubes and incubated at 37 °C for 30 min, after which 5 mL pancreatine-bile solution were added into gastric digest in the centrifuge tubes and incubated for a further 2 h.

The dialysis bags containing the available minerals that has diffused the gastric digest were then rinsed with deionized water carefully dried and weighed. The dried dialysis bag was analysed for mineral content as explained below.

6.3.2.2 Mineral Analysis

Mineral content of the samples (undigested lupin, tempeh and natto samples = total mineral content; dialysis bags from digested samples = available minerals) was measured using a Vista Pro inductively coupled plasma optical emission spectrometer (ICP-OES) (Varian, Palo Alto, USA) at the National Measurement Institute, Perth, Western Australia. Whole seed and de-hulled seed raw lupin, lupin tempeh and lupin

natto (1 g) or the dried dialysis bags from their gastrointestinal digestion were then chemically digested with 3 mL of concentrated HNO_3 plus 3 mL of concentrated HCl in a DEENA automated digestion block (Thomas Cain, Nebraska, USA) at 95 °C for 2 h. After chemical digestion, the sample tubes were made up to 40 mL with distilled water, and solutions were left to settle. Sample supernatants were diluted 5-fold with deionised water before they were analysed using the ICP-OES. Appropriate emission wavelengths that had higher sensitivity and lower interferences were chosen to analyze elements for example wavelengths used to measure Fe, Ca, P and Zn were 238.204 nm, 327.395 nm, 117.434 nm and 213.857 nm, respectively. All results for total minerals and for avaiable minerals were calculated as mg 100 g^{-1} sample (db).

6.4 Statistical Analysis

Data are presented as mean values with standard deviation. Repeated measures in general linear model were used to investigate the main effects of either seed preparation stage or time of fermentation and of seed source (WS, DHS) and their interaction on total and bioavailable minerals. When main effects or interaction were significant, individual means were compared by Bonferroni test. ($P < 0.05$) was considered as significant. IBM SPSS statistics V.24 (IBM Corp. NY, USA) was used for all analyses.

6.5 Results and Discussion

Minerals and trace elements of the raw lupin (WS) are presented in Table 6.1 and these values are compared with mineral values of other commonly consumed legume seeds as reported by Fachmann et al. (2000) (Table 6.2) and USDA (2015) (Table 6.3). Previously, reported mineral values vary very much according to the author (depends on experimental methods, variety of the seed, year of the production and geographical area). For example; Fachmann (2000) reported mineral values of some of important legumes as shown in Table 6.2 and the values are different from the values from (USDA, 2015) Table 6.3. Calcium (Ca) content of raw whole seed lupin reported in the present study (300 mg 100 g^{-1} db) is higher than the value reported in USDA data (176 mg 100 g^{-1} db.). According to USDA data soybean has highest Ca content which is 277 mg 100 g^{-1} db. followed by lupin 176 mg 100 g^{-1} and common bean 123 mg 100 g^{-1}. However, as reported in Fachmann (2000) soybean contains 201 mg 100 g^{-1} of Ca and common bean contains 197 mg 100 g^{-1} of calcium. Therefore, apart from variations such as type of species, variety, geographical area year of production and soil condition lupin contains high content of Ca compared to other legumes.

Table 6.1 Total mineral content (mg/100g db) of Australian sweet lupin as affected by processing stage[1].

Mineral	Source	Mineral content		
		Raw	Soaked	Cooked
Ca	WS	300±10.0^{aA}	286±11.5^{aA}	293±63.5^{aA}
	DHS	126±5.7^{aB}	110±10.0^{aB}	130±10^{aB}
Mg	WS	220±0.0^{aA}	213±5.7^{aA}	176±40.4^{bA}
	DHS	210±10.0^{aA}	186±11.5^{aA}	160±10^{bA}
K	WS	1200±0.0^{aA}	1100±0.0^{bA}	863±193.4^{cA}
	DHS	1500±0.0^{aB}	1023±68.0^{bB}	960±52.9^{cB}
Cu	WS	0.38±0.05^{aA}	0.46±0.01^{aA}	0.51±0.11^{aA}
	DHS	0.46±0.01^{aB}	0.46±0.05^{aA}	0.49±0.03^{aA}
Fe	WS	4.75±0.1^{aA}	4.70±0.12^{aA}	5.20±2.36^{aA}
	DHS	4.4±0.06^{aA}	3.6±0.29^{aA}	3.6±0.26^{aB}
P	WS	350±10^{aA}	345±10^{aA}	310±69.2^{aA}
	DHS	436±5.7^{aB}	390±36.0^{aB}	390±26.4^{aB}
Zn	WS	3.65±0.10^{aA}	3.65±0.06^{aA}	3.40±0.78^{aA}
	DHS	4.20±0.06^{aB}	3.60±0.29^{aB}	4.0±0.26^{aB}
B	WS	1.09±0.1^{aA}	0.46±0.01^{aA}	0.51±0.11^{bA}
	DHS	1.93±0.10^{aB}	1.69±0.13^{aB}	1.52±0.10^{aB}
Mn	WS	1.79±0.08^{aA}	1.71±0.06^{aA}	1.51±0.35^{bA}
	DHS	1.90±0.10^{aB}	1.75±0.15b^{bB}	1.9±0.15^{aA}

[1]Mean ± standard deviation WS, whole lupin seed; DHS, dehulled lupin seed

Values within the same row with different lower case superscript letter denote significant difference ($P<0.05$) using Bonferroni Test

Values at the same processing stage for the same mineral within the columns with different upper case superscript letters denote significant difference ($P<0.05$) using Bonferroni Test

As expressed in Table 6.1 lupin contains very comparable Mg (220 mg 100 g^{-1}) to soybean and other legumes and common bean contains highest (Fachmann et al., 2000). Potassium (K) content of lupin reported in present study (1200 mg 100 g^{-1}) had very close value to soybean (1364 mg 100 g^{-1}) from the other literature (Fachmann et al., 2000). However, USDA data reports that soybean contains higher K content (1797 mg 100 g^{-1}) than lupin (1013 mg 100 g^{-1}) and other legumes. Previously reported data on Cu content of legumes ranged from 0.13 -1.1 mg 100 g^{-1} db and lupin contains (present study) comparative value to soybean and common bean. Iron (Fe) content of legumes in the literature ranged from 4.7-7 mg 100 g^{-1} and lupin reported in present study was very comparable (4.7 mg 100 g^{-1}) to soybean (6.6 mg 100 g^{-1}) and other legumes. Lentil is reported to have the highest Fe content. Phosphorus (P) values for lupin seed of the present study are comparatively lower than as reported in USDA data and lower than P content of soybean reported in both USDA and Fechmann, 2000. As reported in USDA (2015) (Table 6.2) lupin contains (4.75 mg 100 g^{-1}) similar amount of P to soybean (4.89 mg 100 g^{-1}).

Table 6.2. Total mineral content of commonly used other legume seeds compared to lupin (mg 100 g^{-1} db.)

Legume	species	Mineral content						
		Ca	Mg	K	Cu	Fe	P	Zn
Common bean	*Phaseolus vulgaris*	197	250	1483	0.13	7.0	352	3.0
Pea	*Pisum sativum*	96	132	823	0.70	4.8	321	3.5
Chickpea	*Cicer arietnum*	124	155	718	0.85	6.9	252	2,7
Lentils	*Lens culinaris*	71	129	677	0.50	7.5	281	3.2
Soybean	*Glycine max*	201	220	1364	0.83	6.6	649	4.7

Source of data: Fachmann et al. (2000)

Table 6.3 Total mineral content of some legumes compared to lupin (mature, raw seed; lupin species not documented)

Legume	species	Mineral content						
		Ca	Mg	K	Cu	Fe	P	Zn
Bean	*Phaseolus vulgaris*	123	171	1483	na	5.02	352	3.68
Peas	*Pisum sativum*	37	49	823	na	4.82	321	6.99
Chickpeas	*Cicer arietnum*	57	79	718	na	4.31	252	2.76
Lentil	*Lens culinaris*	35	6.51	677	na	6.51	281	3.27
Soybean	*Glycine max*	277	280	1797	na	15.7	704	4.89
Lupins	*Lupinus spp.*	176	198	1013	na	4.36	440	4.75

Source of data: USDA (2015)

6.5.1 The effect of processing on mineral content of Australian sweet lupin

The effects of processing stage (raw, soaked, and cooked) and source of lupin (WS, DHS) on mineral content of lupin seeds are shown in Table 6.1. There was a significant (P<0.05) main effect of source of lupin on Ca content but no effect (P > 0.05) on stage of processing. However, there was no (time x source) interaction (on the Ca content of ASL. The Ca level in WS was signficanly higher than in DHS when compared all processing stages and which supporting prervious reports that Ca is mostly deposited in the hull. As *L.angustifolius* has 25% hull fraction, about ~ 70% of Ca is found in the hull and only ~ 30% in the kernel.

Magnesium content was significantly (P < 0.05) affected by the processing stage. Neither the source of the lupin nor processing stage had main effect or the interaction (processing stages x souce). Magnesium content of raw lupin was significantly (P < 0.05) higher than

cooked lupin and the content of soaked lupin was significantly higher from cooked lupin but there was no significant difference (P > 0.05) between Mg content of soaked compared to raw lupin. However, Mg content of cooked lupin was lower than that of raw and soaked lupin. In contrast to Ca, distribution of Mg in the hull and kernel was similiar at 50% in hull and 50% in kernel.

Both source of lupin seed (WS and DHS) and processing stage (raw, soaked and cooked) had significant main effects (P < 0.05) on K content. Also, there was a significant (P < 0.05) interaction of (processingx source) effect. Whole seed had a significantly higher K content (P < 0.05) than dehulled lupin at all processing stages. This suggest that K is more concentrated in the kernel, as 80% in kernel and 20% in the hull. Present results agree with previous finding Hung et al, (1988). Also, raw lupin had significantly higher (P < 0.05) content than soaked and cooked lupin for both seed types. These findings could be due to K leaching during soaking and cooking processing steps.

Copper content was significantly affected by seed source but there was no effect of processing stage. Dehulled lupin seed had higher Cu content compared to whole seed.

Pospours content had a significant effect of seed source (P < 0.05) while processing stage had no significant effect. The processing x soucre intercation was not noticed. Dehulled lupin seed had significantly higher P content compared to whole seed lupin at all processing stages (P < 0.05).

The Fe content of lupin was not significantly affected either by source or processing method. Also there was no processing x source interaction.

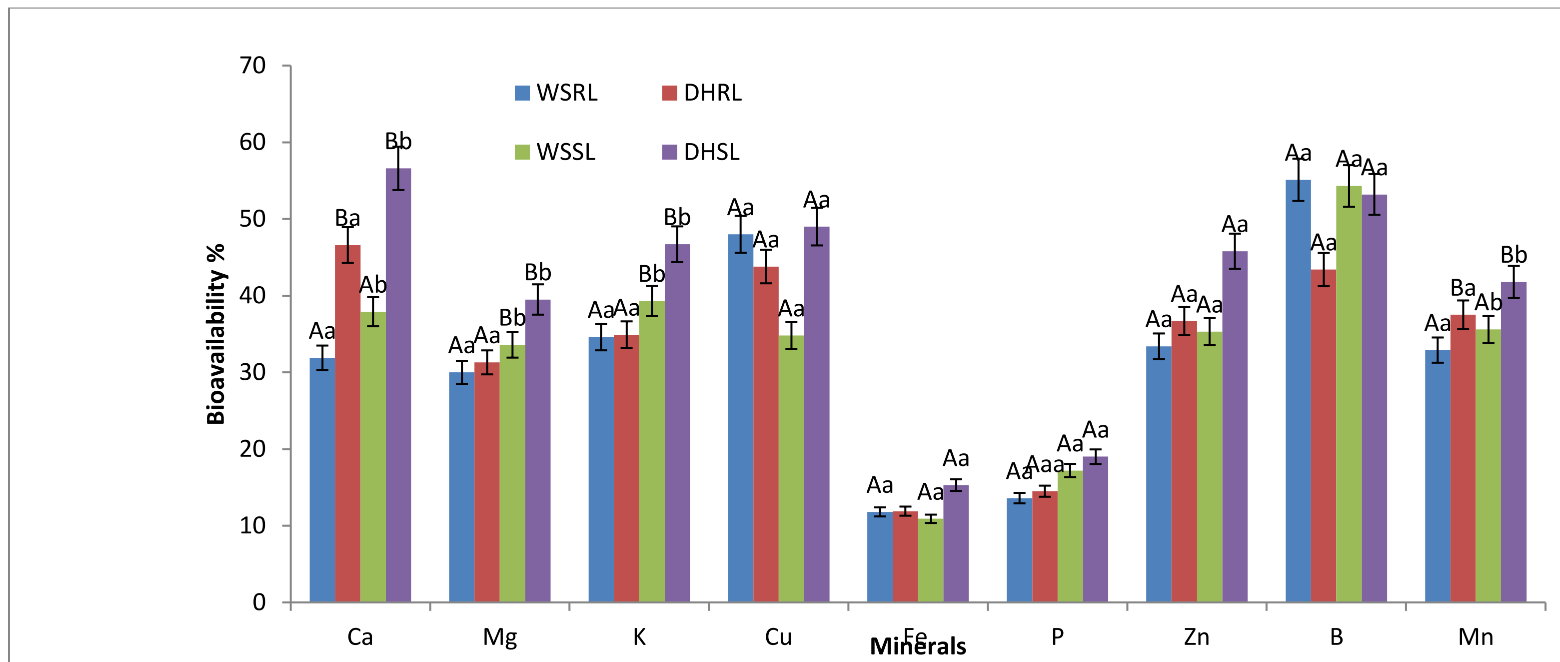
Lupin source had a significant effect ($P < 0.05$) on Zn content of lupin. However, there was no significant effect of processing method on Zn of lupin. Dehulled lupin seed had a significantly higher ($P < 0.05$) Zn content than whole seed at all processing stages.

There was a significant effect of seed source on Manganese (Mn) content of lupin seeds. There was a significant ($P < 0.05$) main effect of processing, however, interaction of processing x source was not evident. In contrast to Zn, Mn content was higher in whole lupin seed at all processing stages. Some species of lupin including *L. albus can* contain higher Mn levels compared to *L. angustifolius* (Hung, Handson, Amenta, Kyle, & Yu, 1987) used in the present study. A high Mn content may be unsuitable for human consumption, since animal studies have indicated that high dietary levels of Mn cause loss of appetite, mild toxicity and reduce growth in pigs, poultry and sheep (Glastone,1962; Hung et al., 1988). Lupin mineral composition data which reported in the present study closely resembles the data reported by (Hung et al., 1988) for *L. angustifolius* except for K and Mn. Potassium content is higher and Mn showed lower values in the present results.

6.5.2 Effect of processing stage on mineral bioavailability of Australian sweet lupin

Figure 6.1 presents the mineral bioavailability of ASL of raw whole seed, raw dehulled seed, soaked whole seed and soaked dehulled seed. Bioavailability of Ca was signifcantly ($P < 0.05$) affected by processing and source. However, there was no interaction of processing x source. Ca bioavailability of soaked lupin was higher than raw lupin and Ca bioavailability of dehulled seed was higher compared to whole seed. Magnesium bioavailability of dehulled soaked was significantly ($P < 0.05$) affected by processing method but source had no effect and there was no interaction between source and processing method. Dehulled soaked had the highest bioavailability and whole seed raw lupin had the lowest bioavailability of Mg. Similarly, K bioavailability was significantly ($P < 0.05$) affected by processing but there was no effect of source. Also, there was no interaction of processing x source. Bioavailability of Cu, Fe, P, Zn, B, had no effect of processing or source and there was interaction. However, Mn had main effect on processing method and source. Food processing can affect mineral binding and availability (Loewus et al,1998). Prolong soaking and may lead to mineral loses as a results of leaching and these losses can differ depending on pH and temperature. There are differences in optimal conditions for phytases degradation between plant species Most cereal phytates have pH optima between 4.5-5.6 but pH optima of some legumes are neutral or alkaline (Loewus et al,1998). Phytates degradation occurred in broad beans at pH 4.5 - 8 at 37 °C highest phytic degradation reported at pH 7 at 55 °C.

1



2

Figure 6.1 Effect of processing on mineral bioavailability of Australian sweet lupin

3 WSRL, whole seed raw lupin; DHRL, Dehulled raw lupin; WSSL, whole seed-soaked lupin; DHSL, Dehulled soaked lupin

4 [1]Mean ± standard deviation

5 Values within a mineral with different lower-case letter denote significant difference ($P < 0.05$) between the same seed source at different processing stage

6 Values within a mineral with different upper case superscript letters denote significant difference ($P < 0.05$) between the two seed sources at the same
7 processing stage

8

6.5.3 Effect of fermentation time on mineral bioavailability of lupin natto

Calcium (Ca) bioavailability of lupin natto was significantly affected by source of lupin. However, there was no significant effect ($P < 0.05$) on fermentation time. Interaction between time x source was non-significant. In a pairwise comparison, there was a significant increase in dehulled lupin seed compared to whole lupin seed. Magnesium (Mg) bioavailability of lupin natto had a significant effect of source but fermentation time did not have significant effect. Also, mineral bioavailability X source did not have significant effect on Mg bioavailability. Potassium (K) bioavailability of lupin natto was not significantly affected by source or fermentation time (there was no main effect). Furthermore, Copper (Cu) bioavailability did not have any significant main effect of source or fermentation time. Similar to most of other mineral bioavailability of lupin natto, Cu bioavailability was not affected by source or fermentation time. Iron (Fe) bioavailability of lupin natto had a significant main effect of source and fermentation time. In pair wise comparison, 48 hours fermented samples had highest ($P < 0.05$) Fe bioavailability compare to unfermented (0 h fermented) samples. Also, Fe bioavailability of 48 h fermented samples were significantly higher ($P < 0.05$) compared to 12 & 36 h fermented lupin natto. The Fe bio availability of dehulled lupin natto was significantly higher than whole seed and 48 h fermented lupin natto (longer the fermentation period higher the bioavailability) had a significant higher Fe bio availability compared to unfermented. Phosphorus (P) bioavailability of lupin natto did not have any significant effect of source and fermentation time. However, in a pair wise comparison, 24 h fermented lupin natto

32 had a significant higher P bioavailability compared to 0 h fermented lupin natto. Also, 48
33 h fermented lupin natto had higher P bio availability compared to 12 h fermented lupin
34 natto. Even though, there was no main effect of fermentation time, 48 h fermented
35 samples had a significantly higher ($P < 0.05$) bioavailability compared to other samples
36 (12 h fermented lupin natto). Zinc bioavailability of lupin natto had a significant ($P < 0.05$)
37 significant main effect of source but there was no significant effect of fermentation time.
38 In pair, wise comparison dehulled lupin seed had significantly higher mineral
39 bioavailability compared whole seed. Similarly, Boron (B) bioavailability of lupin natto had
40 a significant main effect of fermentation time. Boron bioavailability of dehulled lupin natto
41 was significantly ($P < 0.05$) higher than whole seed lupin natto. There was a main effect
42 of source and fermentation time on Mn bioavailability of lupin natto. Lupin natto samples
43 which are fermented up to 12 h had significantly higher Mn bioavailability compared to
44 36 h fermented lupin natto.
45

46 Table 6.4. Effect of fermentation time and source of lupin on mineral bioavailability of lupin natto[1]

47

Mineral	Source	Bioavailability (%)				
		0 h	12 h	24 h	36 h	48 h
Ca	WS	31.98±4.51$^{a, A}$	32.26±4.60$^{a, A}$	29.29±0.37$^{a, A}$	34.40±5.00$^{a, A}$	29.86±4.00$^{a, A}$
	DHS	45.03±3.86$^{a, B}$	49.73±10.71$^{a, B}$	49.88±5.49$^{a, B}$	45.30±3.15$^{a, B}$	41.35±1.90$^{a, B}$
Mg	WS	38.00±3.25^{aA}	41.65±3.74^{bA}	38.20±0.13^{aA}	45.62±3.20^{bA}	42.00±2.00^{aA}
	DHS	40.12±0.00^{aB}	45.37±6.10^{aB}	41.65±5.87^{aB}	33.50±1.15^{aB}	33.06±3.45^{aB}
K	WS	44.00±4.83^{aA}	58.06±17.88^{bA}	44.82±1.33^{bA}	53.88±1.66^{cA}	53.41±6.65^{dA}
	DHS	56.20±2.72^{aA}	53.07±4.50^{aA}	53.85±1.63^{aA}	54.53±3.46^{aA}	51.82±3.90^{aA}
Cu	WS	35.00±5.37^{aA}	36.70±3.25^{aA}	38.80±1.70^{aA}	41.40±4.53^{A}	43.60±3.68^{bA}
	DHS	37.34±2.88^{aA}	38.98±9.24^{aA}	50.20±9.52^{bB}	45.71±2.02^{bA}	42.14±2.16^{aA}
Fe	WS	8.29±0.44^{aA}	9.15±2.04^{aA}	6.57±0.06^{aA}	7.85±1.67^{aA}	8.15±0.16^{aA}
	DHS	11.34±0.37^{aB}	15.46±3.45^{aB}	26.79±7.29^{bB}	16.25±0.78^{aB}	26.14±1.18^{bB}
P	WS	18.03±1.23^{aA}	16.45±0.18^{aA}	15.90±2.55^{aA}	17.24±0.16^{aA}	21.37±2.53^{aA}
	DHS	20.57±2.96^{aA}	22.27±0.67^{aA}	17.09±2.52^{aA}	16.38±0.65^{aA}	18.77±2.07^{aA}
Zn	WS	28.57±3.17^{aA}	32.02±4.40^{aA}	32.03±1.34^{aA}	36.21±4.58^{aA}	34.32±3.44^{aA}
	DHS	40.63±1.59^{aB}	43.75±8.13^{aB}	47.25±6.72^{bB}	38.25±1.06^{aA}	38.38±3.01^{aA}
B	WS	38.63±3.82^{aA}	35.43±1.09^{aA}	39.87±2.07^{aA}	50.06±1.13^{bA}	54.63±2.69^{bA}
	DHS	54.27±6.24^{aA}	72.37±6.51^{bB}	59.08±13.22^{aB}	58.04±0.34^{aB}	53.81±5.12^{aA}
Mn	WS	37.54±4.61^{aA}	36.82±3.65^{A}	34.46±0.82^{aA}	39.68±4.41^{abA}	34.58±3.48^{aA}
	DHS	42.81±0.70^{aA}	51.74±0.45^{bB}	40.73±0.23^{aB}	35.01±0.29^{aB}	36.39±4.95^{aA}

48

49 WS, whole lupin seed; DHS, dehulled lupin seed

50 [1]Mean ± standard deviation

51 [a, b] Values within the same row with different lower case superscript letter denote significant difference ($P < 0.05$) using Bonferroni Test

52 [A, B] Values at the same time point for the same mineral within the columns with different upper case superscript letters denote significant
53 difference ($P < 0.05$) using Bonferroni Test

6.5.4 Effect of fermentation time on mineral bioavailability of lupin tempeh

The effect of fermentation time on minerals and trace elements bioavailability was statistically analyzed using general linear model, repeated measures. Source of lupin had a significant ($P < 0.05$) main effect on Ca bioavailability of lupin tempeh. However, there was no overall significant effect of fermentation time on Ca bioavailability of lupin tempeh. In pairwise comparison, there was no significant effect of fermentation time on Ca bioavailability.

In similar analysis, source of lupin and fermentation time did not have significant main effect on Mg bioavailability of lupin tempeh. However, in pair wise comparison, 12 h fermented lupin natto had significantly ($P < 0.05$) higher Mg bioavailability compared to 0 h fermented natto samples.

Potassium bioavailability was significantly ($P < 0.05$) affected by fermentation time but source of lupin had no significant effect on K bio availability. In a pair wise comparison, 12 h, 24 h and 36 h fermented lupin tempeh had significantly higher K bioavailability compare to unfermented or 0 h fermented lupin tempeh. However, 48 h fermented tempeh was not significantly different from 0 h fermented tempeh.

Copper bioavailability of lupin tempeh was significantly ($P < 0.05$) affected by source and fermentation time. In pair wise comparison, 48 h fermented lupin tempeh had significantly ($P < 0.05$) higher Cu bioavailability compared to 0 h fermented lupin tempeh. Fermented lupin tempeh which was fermented up to 24 h had significantly lower Cu bioavailability compared to 36 & 48 h fermented lupin tempeh.

77 There was no significant effect of source and fermentation time on Fe availability of lupin
78 tempeh. However, Min_bio X Source had significant (*P*<0.05) effect on Fe bioavailability.
79 In pairwise comparison 36 h fermented lupin tempeh was significantly higher than 24 h
80 fermented samples.
81 There was a main effect of source and fermentation time on P bioavailability of lupin
82 tempeh. In pair wise comparison, 24, 36 and 48 h fermented samples were significantly
83 higher P bioavailability compared to 12 h fermented lupin tempeh.
84 Zinc bioavailability of lupin tempeh was significantly (*P* < 0.05) affected by source and
85 fermentation. In pair wise comparison, 12 h fermented lupin tempeh was significantly
86 lower than 36h and 48h hours fermented lupin tempeh. Fermented lupin tempeh samples
87 which were fermented up to 36 and 48 h had significantly (*P* < 0.05) higher Zn
88 bioavailability compare to 12 h fermented lupin tempeh. Fermented lupin samples (until
89 48 h) had significantly (*P* < 0.05) higher Zn bioavailability compared to12 and 24 h
90 fermented lupin tempeh.
91 There was no main effect of source and fermentation time on Mn bio availability. Also, in
92 pair wise comparison 24 h fermented lupin tempeh (dehulled) was significantly (*P* < 0.05)
93 higher from 36 h fermented lupin tempeh (Table 6.4).
94 Germination reduced the amount of phenolic compounds while natural fermentation was
95 found to increase certain phenolic compounds (Frias et al., 2005). No change of mineral
96 bioavailability of some minerals during fermentation can be explained by the above
97 concept. Also these polyphenolic compounds have strong inhibitory activity on Fe
98 absorption. The reason for not getting expected increase in iron bioavailability may be
99 due to the increase of polyphenolic compounds. Furthermore, Ca can inhibit Fe

100 absorption. Lupin seed has high Ca therefore there can be some interference and
101 reduced iron absorption. Also, Phytic acid removed soybean showed inhibitory effect on
102 iron absorption which may be due to inhibitory peptide binding Fe (Lynch et al,1994).
103 Adding phytase to soybean based infant formula increased Fe absorption significantly but
104 even after removing phytates soybean had some Fe inhibitory effect. This is due to the
105 presence of Fe-binding peptides and absorption ranged from 42%- 54% (Davidson,
106 1994). However, present study results showed low absorption compared to above data
107 (8.29-26.79%).
108 In plant cell walls Mg may be bound by lignin but not by pectin. The lower absorption of
109 Mg in bran is probably due to its higher phytate content, since phytic acid has been found
110 to bind Mg and reduce its bioavailability (Seelig ,1980). High amount of Ca, P , fibre and
111 phytates appear to inhibit Mn absorption and Mn-Fe interactions have been
112 demonstrated. Absorption is < 5% but present experiment it is around 30-40%.
113 Manganese from soy protein was found to be better absorbed than from casein protein
114 (Lee & Johnson, 1989).
115 Several dietary constituents have been suggested to have an influence on Mg
116 bioavailability. Enhances include protein and amino acid and inhibitors include
117 phosphates, phytates and dietary fibre. The influence of different processing methods
118 including fermentation on mineral and trace elements and its bioavailability was
119 investigated by measuring mineral dialysability. Different types of protein and peptide
120 has different influence on mineral bioavailability. This may have influenced on the raw
121 lupin, lupin natto and tempeh samples variability. The enhancement of animal tissue on
122 Fe and Zn absorption has come to be termed the “meat effect” because this effect results

123 from a variety of meat products (Fransica, 1997). The exact mechanism is not known but
124 may be related to the protein of the meat it is believed that peptides released during
125 proteolytic digestion can prevent the formation of iron complexes (Poliz & Clydes
126 dale,1988) or meat contain one or more factors that solubilise iron independent of
127 proteolytic digestion (Carpenter & Marhoone,1989). The highest phytate degradation can
128 be achieved by providing optimal conditions for the degradation process. Legumes have
129 highest phytase degradation in neutral and or alkaline pH. Once phytates and
130 polyphenols is degraded, legumes would become good source of Fe and Zn as the
131 content of these minerals are high (Sandberg, 2002). However, fermentation is a paradox
132 when it comes to mineral bioavailability as, while it reduces some agents which can bind
133 mineral (e.g phytic acid), it increases some other factors which reduces mineral
134 bioavailability (polyphenols).
135
136
137

138 Table 6.5. Effect of fermentation time and source of lupin on mineral bioavailability of lupin tempeh[1]

139

Mineral	Source	Bioavailability (%)				
		0 h	12 h	24 h	36 h	48 h
Ca	WS	29.39±0.27^{aA}	26.30±0.28^{aA}	27.35±1.77^{aA}	25.54±3.22^{aA}	28.07±1.08^{aA}
	DHS	43.28±2.15^{aB}	42.88±3.54^{aB}	42.96±0.28^{aB}	49.57±1.36^{aB}	41.77±4.46^{aB}
Mg	WS	34.01±0.81^{aA}	29.72±0.13^{aA}	30.85±1.73^{aA}	29.92±2.97^{aA}	32.96±3.30^{aA}
	DHS	33.53±1.46^{aA}	33.03±0.75^{aA}	31.84±0.66^{aA}	32.91±0.22^{aA}	33.05±7.00^{aA}
K	WS	50.04±3.15^{aA}	37.73±0.19^{aA}	37.40±1.39^{aA}	37.34±0.74^{aA}	39.36±2.32^{aA}
	DHS	40.06±0.44^{aA}	36.63±4.07^{aA}	36.56±1.68^{aA}	39.94±2.38^{aA}	39.75±0.35^{aA}
Cu	WS	18.49±5.72^{aA}	16.67±2.69^{aA}	15.24±0.45^{aA}	21.42±1.80^{bA}	24.76±6.06^{bA}
	DHS	22.07±0.62^{aB}	26.15±8.27^{bB}	26.93±2.18^{bB}	38.96±2.96^{bB}	39.29±0.31^{bB}
Fe	WS	13.82±0.76^{aA}	13.14±2.96^{aA}	9.92±0.44^{aA}	9.55±0.56^{bA}	12.01±2.19^{aA}
	DHS	8.21±0.96^{aA}	9.53±0.00^{aA}	11.78±1.03^{aA}	14.78±1.03^{bA}	10.22±0.28^{aA}
P	WS	15.92±0.37^{aA}	12.68±1.97^{aA}	12.58±0.11^{bA}	12.35±0.88^{cA}	15.50±1.00^{dA}
	DHS	11.85±0.40^{bB}	10.29±0.08^{aB}	17.15±0.14^{bB}	20.17±0.64^{bB}	20.71±0.91^{bB}
Zn	WS	30.53±1.86^{aA}	25.94±0.52^{aA}	26.15±1.71^{aA}	30.26±0.00^{aA}	31.71±1.68^{aA}
	DHS	33.20±0.74^{aB}	34.36±1.07^{aB}	33.84±0.16^{aB}	36.51±0.00^{aB}	38.78±1.39^{bB}
B	WS	79.06±10.29^{aA}	57.23±7.33^{aA}	52.63±1.07^{aA}	44.28±1.51^{aA}	61.25±15.41^{aA}
	DHS	42.82±1.16^{aA}	41.89±0.16^{aA}	43.57±0.91^{aA}	61.57±20.00^{aA}	60.46±0.65^{aA}
Mn	WS	38.80±1.70^{aA}	34.32±0.79^{aA}	36.16±0.62^{bA}	32.82±4.27^{bA}	36.60±4.53^{bA}
	DHS	34.64±2.46^{aA}	35.47±1.48^{aA}	30.02±1.17^{bA}	33.19±1.21^{aA}	33.88±5.76^{aA}

140

141 WS, whole lupin seed; DHS, dehulled lupin seed

142 [1]Mean ± standard deviation

143 $^{a, b}$ Values within the same row with different lower case superscript letter denote significant difference (P<0.05) using Bonferroni Test

144 $^{A, B}$ Values at the same time point for the same mineral within the columns with different upper case superscript letters denote significant
145 difference (*P* < 0.05) using Bonferroni Test

146

147
6.6 Conclusion

148 As presented in Table 6.1 and 6.2 lupin is rich in essential minerals and trace elements.
149 Also, its mineral content was comparable to commonly consumed other legumes.
150 There was a significant effect (P<0.05) of processing methods (unprocessed, soaked
151 and boiled) on the bioavailability of Ca, K, Cu and Zn of ASL. In contrast, bioavailability
152 of Mg, Fe, P, B and Mn were not affected by processing methods. Bio availabilities of
153 some minerals of lupin tempeh were significantly (P<0.05) increased by fermentation
154 time (Cu, P, Zn) and source (Ca, Cu, P and Zn) of lupin. The Cu content of 36 h and 48
155 h fermented WS lupin tempeh, was significantly ($P < 0.05$) higher than WS 0 h
156 (unfermented) lupin tempeh. Bioavailability of Zn was increased with fermentation time
157 and dehulled seed had significantly higher ($P < 0.05$) bioavailability in lupin tempeh. Iron
158 bioavailability lupin natto was increased with fermentation time. Dehulled lupin natto had
159 highest bioavailability at 24 h and 48 h fermentation. Bioavailability of Zn had a
160 significant effect of source and dehulled lupin natto had significant higher value than
161 whole seed. Zinc bioavailability showed interaction with fermentation time and source.
162 Zn bioavailability of whole seed lupin natto was highest at 36 h and dehulled lupin natto
163 highest value at 12 h. It was expected that there will be a significant increase in mineral
164 bio availability due to fermentation, however, it was not obvious in most of the minerals
165 tested on the present study. This may be due to other biochemical changes which
166 happens during fermentation i.e. increase in polyphenols and peptides to compete with
167 minerals There was a paradox effect of fermentation on mineral bioavailability of lupin.

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169

170

CHAPTER 7

171

172

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

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174 Australian sweet lupin (ASL) has the potential as a functional food and a substitute for
175 soybean in food product applications due to its nutritional composition and functional
176 similarities. In this investigation, an attempt was made to improve the functional attributes
177 of lupin through the process of solid-state fermentation. The results presented in this
178 thesis have reported on the production of lupin based natto and tempeh; two commonly
179 consumed Asian foods that have high consumer acceptance in the region. The findings
180 demonstrate that lupin can be used to produce these traditional Asian fermented foods.
181 Furthermore, physical, organoleptic and chemical characteristics of lupin natto and
182 tempeh were found to be highly comparable to natto and tempeh derived from soybean
183 and in some instances even improved upon. The phytoestrogens genistein and daidzein
184 content in lupin was found to be low compared to soybean, but the increase in these
185 compounds due to fermentation, still well safer levels for human consumption. There were
186 some improvements in *in vitro* mineral bioavailability as a result of fermentation, however,
187 in vivo trials are now needed to confirm these findings. The first aim was to determine the
188 effect of seed source (WS & DHS), seed processing methods and fermentation time on
189 physical, organoleptic and chemical characteristics of lupin natto and lupin tempeh. The
190 output of chapter 3 indicates that lupin can be used for preparing high quality natto and
191 tempeh. Also, results showed that physical, organoleptic and chemical characteristics of
192 lupin natto and tempeh were significantly improved by ($P < 0.05$) by fermentation time,

193 source and microbial strain. Natto fermented up to 24 h and tempeh fermented to 48 h
194 were identified as the optimum fermentation time for preparing each product. Lupin natto
195 and tempeh prepared from dehulled lupin seed was significantly ($P < 0.05$) better over
196 whole seed in quality attributes. Also, tempeh prepared from Indonesian commercial
197 starter showed better ($P<0.05$) quality attributes compared to tempeh from USA
198 commercial starter. Natto prepared from ATCC 15245 showed significantly high ($P < 0.05$)
199 quality attributes in comparison to lupin natto prepared from ATCC 6633. Furthermore,
200 inoculum level 0.2 (mg 100 g^{-1}) was identified as suit able inoculum level for lupin tempeh
201 preparation at the present experimental conditions (37 °C, 85% humidity). Bacteria count
202 containing (10^{4}-10^{6} cells g^{-1}) of the starter was the best inoculum level for preparing lupin
203 natto. These results highlight the need for selecting optimal fermentation period, source
204 of lupin and microbial strain for the maximum final product quality. This information
205 advances the gap in the knowledge in the field of lupin natto and tempeh and is the first
206 report on production of lupin natto from ASL. There were number of studies on preparation
207 and nutritional composition of lupin tempeh (Agosin et al., 1989; Fudiyansyah et al.,
208 1995a; Ortega-david & Rodríguez-stouvenel, 2013). However, in this study present wide
209 range of new scientific data such as physical, organoleptic and chimerical characteristics
210 of lupin tempeh as affected by fermentation period, source and microbial strain.
211 Furthermore, preparation of lupin natto, and its nutrition compositional analysis and other
212 physical, organoleptic and chemical characteristics were reported at the first time.
213 Although, the organoleptic characteristics findings reported in this thesis have used a less
214 number of participants than optimal, the outcome have been supported with physical and

chemical tests. Therefore, more in depth studies on sensory evaluation is required in the future.

Findings of chapter 4 indicates that fermentation time and source had a significant ($P < 0.05$) effect on nutritional composition and protein digestibility of during lupin natto and lupin tempeh production. Therefore, the second aim of this thesis was achieved. Dehulled lupin natto which was fermented up to 24 h had highest protein, fat, soluble dietary fibre (SDF) and *in vitro* protein digestibility (IVPD) among all the lupin natto samples tested. On the other hand, 48 h fermented dehulled lupin tempeh had significantly higher ($P < 0.05$) protein, fat, SDF and IVPD among the tempeh samples. Also, the insoluble dietary fibre (IDF) content of dehulled lupin (in lupin natto and tempeh) was significantly reduced during fermentation period and in contrast, SDF content was proportionately increased. Therefore, the *B. subtilis* fermentation of lupin natto and the *R. oligosporus* fermentation of lupin tempeh of ASL improved the nutritional composition, digestibility and fibre profile which suggest increased functional food effects compared to the unfermented lupins.

The third aim of this project was to study the effect of different seed source (whole seed and de-hulled seed), and fermentation time (0-72 h) using a standardised method (from aim 1) on genistein and daidzein content. Findings of chapter 5 revealed that this was achieved and ASL contained comparatively lower values of phytoestrogen isoflavone compounds genistein and daidzein compared to soybean and some other *Lupinus spp.* Genistein and daidzein content decreased with soaking and cooking due most probably to leaching and heat destruction. However, fermentation increased the amount of genistein and daidzein over the fermentation time and the concentration of both

238 compounds peaked at 36 h in lupin tempeh and 48 h in lupin natto. However, this increase
239 is not of any negative health concern as the maximum levels of these phytoestrogens in
240 both final products were within safe limits. Therefore, the lupin natto and lupin tempeh
241 appears are safe for human consumption from this perspective.
242 The fourth aim of this thesis (Chapter 6) was assessing the mineral composition, and
243 *in vitro* bioavailability of lupin seeds and their natto and tempeh as affected by seed
244 source and fermentation time. Results of chapter 6 indicated that the aim was
245 accomplished. As summarized and described in Table 6.1 and 6.2 raw lupin seed was
246 known to be rich in essential minerals and trace elements. Also, its mineral content was
247 comparable to other commonly consumed legumes. Results of this chapter found that
248 there was a significant increase on the bioavailability of Ca, K, Cu and Zn content of ASL
249 with processing stage (unprocessed, soaked and boiled). In contrast, Mg, Fe, P, B and
250 Mn bioavailability were not affected by processing methods. Bio availabilities of some
251 minerals (Cu, P, Zn) of lupin tempeh were significantly ($P < 0.05$) increased by
252 fermentation time. In addition, some minerals (Ca, Cu, P and Zn) were more bioavailable
253 in lupin tempeh from DHS than from WS. Copper content of 36 h and 48 h fermented WS
254 lupin tempeh was significantly ($P < 0.05$) higher than WS 0 h (unfermented) lupin tempeh.
255 Bioavailability of Zn was increased with fermentation time and dehulled seed had
256 significantly higher ($P < 0.05$) Zn than whole seed lupin tempeh. Fe bioavailability in lupin
257 natto was increased with fermentation time. Dehulled lupin natto had highest
258 bioavailability at 24 h and 48 h fermentation. Bioavailability of Zn was significantly affected
259 of source and dehulled lupin natto had significant higher values than whole seed. Whole
260 seed lupin natto had maximum Zn bioavailability at 36 h and dehulled lupin natto's highest

value was at 12 h. Fermentation did not increase the bioavailability of all of the minerals. This may have been due to other biochemical changes which occured during fermentation i.e. the increase in soluble dietary fibre may have had a negative effect on the bioavailability of some minerals.

Key Findings:

The key outputs of this PhD thesis are new knowledge and information on:

1. Laboratory scale production of lupin natto for the first time (characterization of preparation of natto from whole lupin seed and dehulled seed) and analysis of physical, some organoleptic and chemical characteristics of lupin natto.
2. Comparison of nutritional composition (including fibre profile) and protein digestibility of ASL, lupin natto (WS & DHS) and lupin tempeh (WS & DHS).
3. Effect of fermentation time and seed source on phytoestrogens, daidzein and genistein content in ASL natto and lupin tempeh (WS & DHS). To my knowledge, this is the first report in the literature on daidzein and genistein of ASL lupin natto and lupin tempeh.
4. Mineral composition of ASL, lupin natto and lupin tempeh and effect of fermentation and seed source on the mineral bioavailability.

Suggested areas for future directions

The overall improvements in nutritional composition and increase in protein digestibility of ASL from raw seed to lupin natto and tempeh reported in this thesis support the consumption of fermented lupin natto and tempeh as healthy foods. Improvements in

285 mineral bio availability that were found in present study were also promising, but were
286 determined *in vitro*; therefore, conducting in *vivo* studies to further investigate is
287 suggested for future studies.
288 When studying fibre profile, only IDF, SDF and TDF were analysed in present study. In
289 experimental procedure to measure SDF, there were two portions of this fraction i.e.
290 SDFP (soluble dietary fibre precipitate in 78% ethanol) and SDFS (soluble dietary fibre
291 soluble in 78% ethanol). This fraction of SDFS contains oligosaccharide portion of lupin
292 and analysing whole dietary fibre profile including SDFP is recommended in future
293 studies.
294 Also, among phytoestrogenic isoflavone compound daidzein and genistein were studied
295 in this project. However, it is worthwhile next to study whole isoflavone profile including
296 daidzin and genistin and their conversion to genistein and daidzein during fermentation
297 of ASL in a future study.
298 In this thesis, the sensory acceptability of the lupin natto and tempeh was not evaluated
299 in detail (e.g. using a consumer panel), therefore this is important future work that needs
300 to be done.
301 Lastly, future randomized controlled clinical trials could test whether the *in vitro*
302 performance of lupin natto and tempeh transcribes to measurable health benefits for
303 human health. This would confirm the functional benefits of fermented lupin in the
304 prevention of chronic diseases.
305
306

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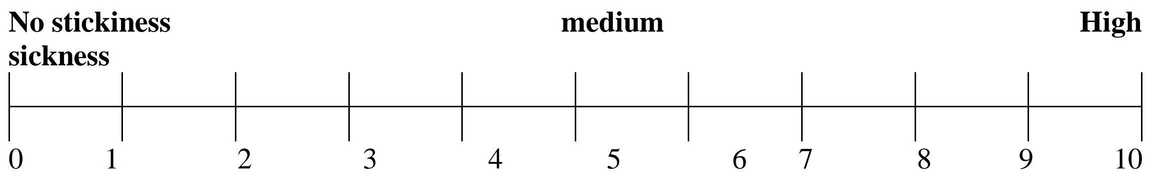
Appendices

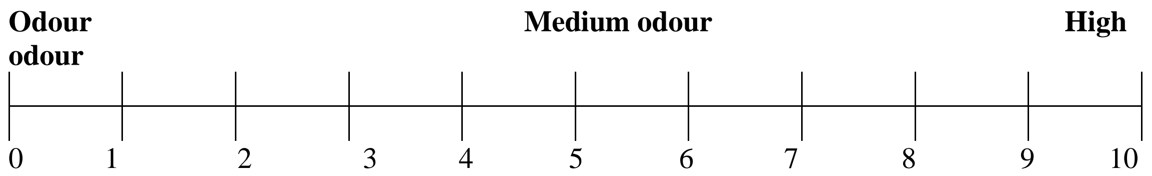
Appendix 1

Visual Analogue Scale Questionnaire – natto

Date ……………………….
Product No ……………………….



0 as off and 10 as perfect



0 as perfect and 10 as perfect

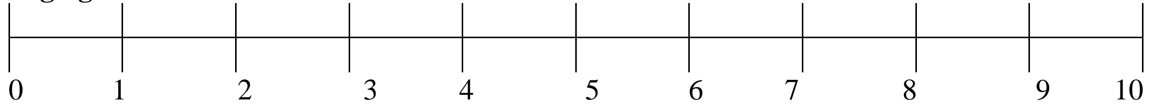
Appendix 2

Visual Analogue Scale Questionnaire – tempeh

Date …………………

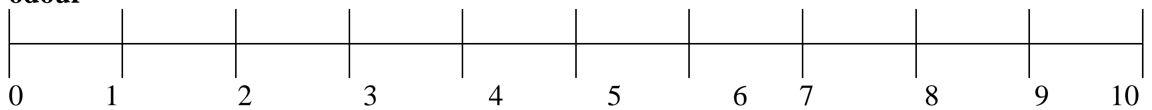
Product No …………………

No mycelium growth **Medium Growth**
High growth



0 as off and 10 as perfect

Odour **Medium Odour** **High**
odour



0 as perfect and 10 as off

Appendix 3

Mycelium growth on lupin tempeh[1] as scored by evaluators using in Visual Analogue Scale (VAS) questionnaire over the fermentation period; effect of inoculum type

Fermentation time (hr)	VAS score mycelium growth (effect of inoculum type)[2]			
	ISC		USC	
	WS	DHS	WS	DHS
0	0±0	0+0	0+0	0+0
6	0+0	0+0	0+0	0+0
12	0+0	0+0	0+0	0+0
18	0.5+0.2	0+0	0.5+0.1	0.5+0.3
24	5+0.1	0.5+0.1	5+0	5+0
30	7.5+0.3	5+0.2	7.5+0	7.5+0.3
36	8+0.4	7.5+0.4	8+0.5	8+0.5
42	9+0.5	10+0.4	8+0.3	8.5+0.3
48	10+0.2	8.5+0	9+0.2	9+0.2
54	9+0.3	8+0.3	8.5+0.5	8.5+0.4
60	9+0.2	8+0.2	8.5+0.3	8.5+0.5
66	8.5+0.3	7+0.5	8+0.3	8+0.4
72	8+0.3	7+0.4	8+0.4	8+0.1
78	5+0.1	5+0.3	5+0.2	5+0.2
84	5+0.3	5+0.2	5+0.1	5+0.3
90	3+0.1	3+0.1	3+0.1	3+0.1

Appendix 4

Mycelium growth on lupin tempeh[1] as scored by evaluators using in house Visual Analogue Scale (VAS) questionnaire over the fermentation period; effect of inoculum level

Fermentation time (h)	VAS score of mycelium growth Inoculums level (mg 100 g^{-1})					
	0.1		0.2		0.3	
	WS	DHS	WS	DHS	WS	DHS
0	0	0	0	0	0	0
6	0	0	0	0	0	0
12	0	0	0	0	0.5	0.5
18	0	0	0.5	0	5	5
24	0.5	0	5	0.5	7.5	7.5
30	5	0	7.5	5	8	8
36	7.5	0.5	8	7.5	9	9
42	8	1	9	9	10	10
48	9	3	10	10	9	9
54	9	5	9	6	9	9
60	8.5	6	9	5	8.5	8.5
66	8	6	8.5	3	8	8
72	7.5	4	8	3	5	5
78	5	3	5	3	3	3
84	5	3	5	3	3	3
90	3	3	3	3	3	3

[1] (ISC, Indonesian commercial stater, 34 °C, Pressure cooked lupin); WS, Whole seed, DHS, De-hulled seed; at 37 °C,

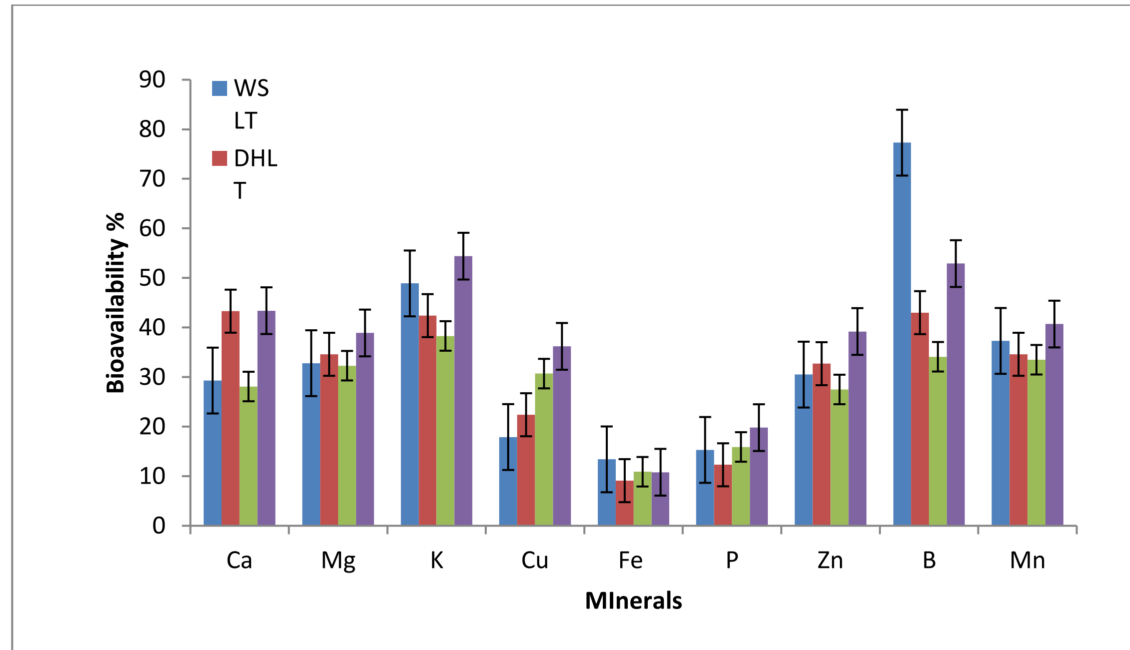
Score,0= no growth, 10= thick mycelium growth covering the substrate, +=0.5

Appendix 5

Mycelium growth on lupin tempeh1 as scored by the evaluators using in house Visual Analogue Scale (VAS) questionnaire over the fermentation period; effect of seed pre-cooking method

Fermentation time	Mycelium growth			
	Boiling		Pressure cooking	
	WS	DHS	WS	DHS
0	0	0	0	0
6	0	0	0	0
12	0	0	0	0
18	0	0	0.5	0.5
24	0.5	0.5	1	1
30	5	5	5	5
36	7.5	7.5	7.5	7.5
42	8	8	8	8
48	9	9	10	10
54	9	9	9	9
60	9	9	9	9
66	8.5	8.5	8.5	8.5
72	8	8	8	8
78	5	5	5	5
84	5	5	5	5
90	3	3	3	3

[1] ISC, Indonesian commercial stater, 34 °C, Pressure cooked lupin; WS, Whole seed, DHS, De-hulled seed; at 37 °C, Score,0= no growth, 10= thick mycelium growth covering the substrate.



Effect of processing on mineral bioavailability of dehulled Australian sweet lupin
WSLT, whole seed lupin tempeh; DHLT, Dehulled lupin tempeh; WSLN, whole seed lupin natto; DHLN, Dehulled lupin natto