Soaking and fermentation of Lupin seeds to improve their nutritional properties

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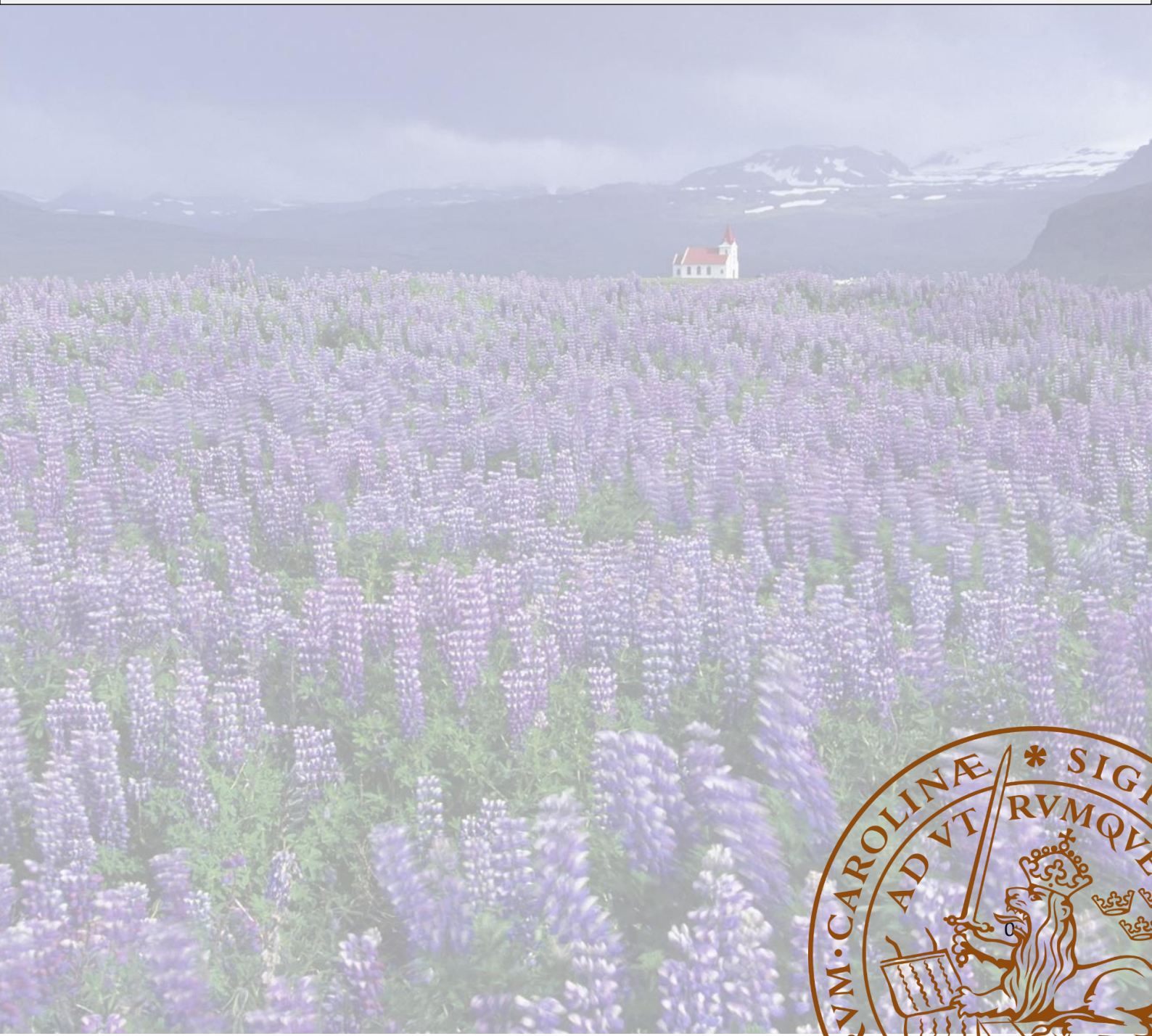

Soaking and fermentation of Lupin seeds to improve their nutritional properties

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For all the people that supported me during my thesis and made sure I had a great time.

I want to say to everyone in the FIKA group

Thanks

Furthermore, I would like to express my sincere appreciation to my supervisor Dr. Claudia Lazarte and my examination Prof. Yvonne Granfeldt for giving me this opportunity to dive deeper into this interesting subject.

I've learned a lot and could not wish for a better ending of my Master's

"The beautiful thing about learning is that no one can take it away from you."

~ B.B. King ~

Popular summary

While the trend nowadays increases the demand for new plant-based products, so is the demand to find new high nutritional food sources. One of these high-nutritional food sources and upcoming legumes is called Lupin. The Lupin seeds have a low carbohydrate, high protein, and high fiber content what makes them an attractive new food source. For many years people that live in all levels of society are fighting against mineral deficiencies. Especially people that have grains and legumes as their staple food source. Like many other legumes, Lupin contains nutrient absorption inhibitors such as phytate that decrease the bioavailability of several minerals. Lupin seeds also contain phenolic components, which are reported to promote anti-inflammatory conditions and due to their anti-oxidation effect they have the ability to prevent cellular damage through free-radical oxidation reactions. For this reason, the aim of this study was to investigate if it was possible to reduce this mineral inhibitor by dehulling, soaking and fermentation of the seeds, in addition to increase the total phenolic content and thus its nutritional profile.

To reduce the phytate, three different methods were compared. In the first method, Lupin seeds were removed from their coat (dehulled) and subjected to a 24-hour soaking period followed by a 24-hour fermentation. In the second method, dehulled seeds were only subjected to a 24-hour fermentation. Where in the third method Lupin seeds were subjected to a 24-hour soaking period and 24-hour fermentation with their coat. None of these methods were able to significantly reduce the phytate content. Where only a combination of dehulling and soaking showed to reduce the phytate content significantly. On the other hand, all 3 methods showed an 3 fold increase in total phenolic content.

Although there are a lot of variables that can have influence on the phytate content: type of bacteria used during the fermentation, initial concentration of phytate reducing enzymes, and the different types of Lupin seeds are just 3 of them. Changing these parameters can result in a decrease of phytate and can thereby have a positive effect on the mineral absorption and mineral deficiencies. In addition, the higher concentration of phenolic compounds in the fermented lupin seeds, presents a potential nutritional improvement in terms of antioxidants found in Lupin seeds and a study on their bioavailability and health effects is warranted.

Abstract

Lupin seeds contain like many other grains and legumes the mineral inhibitor phytate. The presence of this anti-nutrient decreases the bioavailability of minerals such as zinc and iron. Especially people that live in areas where grains and legumes are the staple food source, as well as vegan and vegetarian groups, may be more vulnerable to mineral deficiencies. The presence of phenolic compounds in food can prevent cellular damage and act as an anti-oxidant. During this study, different methods were investigated to reduce the phytate content alongside with an increase of total phenolic content, thereby increasing its nutritional profile. By dehulling, soaking and fermenting the Lupin seeds the possible reduction of phytate and increase in total phenolic content were analyzed.

The seeds of the *Lupin angustifolius* plant have, with their low carbohydrate (<2%), high protein (36%) and fiber (35%) content, a strong nutritional profile that is comparable to other legumes like soybean and lentils. Not only the nutritional profile but also the ability to grown at many geographic locations, and the ability to convert atmospheric nitrogen into more usable forms that improve the soil quality makes it an excellent growing crop with many benefits.

The Lupin seeds were bought from a local farmer in Skåne and divided into three batches. The seeds in method 1 were dehulled and submerged for 24-hour in water with a ratio of 1:3 (w/v) containing 0.5% $NaCO_3$, followed by a 24-hour fermentation (37°C) with *Lactobacillus plantarum 299v* (1%). The seeds in method 2 ware dehulled and subjected to a 24-hour fermentation (37°C) with *Lactobacillus plantarum 299v* (1%). Where in method 3 whole lupin seeds were subjected to a 24-hour soaking period (ratio 1:3 w/v, 0.5% $NaCO_3$) and 24-hour fermentation (37°C) with *Lactobacillus plantarum 299v* (1%).

During the fermentation the pH, TTA (titratable acidity), protein, phytate, and total phenolic content were determined every 3 hours until 12 hours, and 24 hours after initial start of the fermentation. Neither of the 3 methods were able to reduce the phytate content significantly ($p=>0.05$). Where results from ANOVA showed that only a combination of dehulling and soaking reduced the phytate content significant ($p=<0.05$) with 19%. On the other hand, the initial total phenolic content increased on average 3 fold, all the 3 methods showed to be significant ($p=<0.05$). The seeds that were dehulled, soaked and fermented (method 1) showed with 24% the biggest increase of phenolic compounds, the seeds that underwent only the dehulling and fermentation process (method 2) showed the smallest increase of 21%.

According to this study, a combination of dehulling, soaking and fermentation does not significantly reduce the phytate content. Whereas a 3 fold increase is measured of total phenolic content after a 24-hour fermentation period.

For further research it is possible to use a different Lactobacillus species and soak the seeds at a temperature that is more in line with the optimum temperature for phytase activation. Further the phytase activity can be measured throughout the entire process to follow its changes.

Abbreviations

DM	Dry matter
FAO	Food and Agriculture Organization of the United Nations
FTU	Phytase unites; expressed as 1 μmol of phosphate liberated per minute.
IP3	Inositol Triphosphate
IP4	Inositol Tetraphosphate
IP5	Inositol Pentaphosphate
IP6	Inositol Hexaphosphate
IZiNCG	The International Zinc Nutrition Consultative Group
LAB	Lactic acid bacteria
MRS	De Man, Rogosa en Sharpe-agar
NSP	non starch polysaccharides
ROS	Reactive oxygen species
TPC	Total phenolic content

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1. Introduction

Mineral malnutrition is a problem that is not only occurring in third world countries, it's a global problem that also affects industrialized countries. Infants, children and woman at childbearing age are mostly affected by this global problem (Dewan, 2018). Since legumes and whole grains are a good source of carbohydrates, dietary fiber, vitamins, minerals, trace-elements and numerous bioactive compounds, authorities and health organizations recommend the consumption of them frequently (NHMRC , 2013).

However, whole grains and legumes so also Lupin may increase the intake of minerals it also increases the intake of anti-nutrients such as phytate. Phytate is a salt that is formed when phytic acid binds with divalent minerals such as iron, zinc and calcium. Phytates are considered anti-nutrients for their ability to binds minerals such as iron and zinc and thereby reduces their bioavailability, what contributes to mineral malnutrition (FAO, IZINCG, 2018). The recommendation from the authorities to decrease meat intake, the increasing population of vegans and vegetarians, and the current trend to shift from an animal-based diet to a plant-based diet may have an influence on mineral malnutrition (Schlemmer, Frølich, Prieto, & Grases, 2009). This forces companies to develop new products and to find innovative alternatives to existing products to penetrate this growing market. One of these new promising legume species is Lupin, this legume plant consist of more than 450 species and is attractive due to their GMO free status and nutritional profile (Karam, Singh, Kamphuis, & Nelson, 2019). Where some Lupin species have been bred for their ornamental value, others have been a traditional snack in the Andean and Mediterranean region. Lupin (*Lupinus angustifolius*) seeds have with their high fiber and high protein content a strong nutritional profile that is comparable to other beans/legumes such as lentils, chickpea and soy (Lupin Foods Australia, 2013). Nowadays this narrow blue leaved, sweet lupin is mostly produced in Australia (80% of the global production) and in smaller amounts in Europe. Most of this sweet variety of Lupin is used for animal feed and rarely for human consumption, this is due to its high non-starch polysaccharides (NSP), oligosaccharide, secondary plant metabolites content, bitter taste, and their low protein digestibility (70-80% digestibility when compared to proteins from a meat source) (Chin, et al., 2019). Although it is used mostly in animal feed, sweet lupin is a promising new protein source that can be grown in the Nordic countries. The fact that this legume does not need a Mediterranean climate to grow, reduces the transportation cost for production companies located in Scandinavia (Australian government, 2013).

Fermentation with lactic acid bacteria has been shown to be a good strategy to decrease the initial phytate content in legumes and cereals (Fritsch, Vogel, & Toelstede, 2015). Further has lactic fermentation shown to reduce tannin content, which are known to be anti-nutrients and contribute to the bitter taste of Lupin seeds (Lamy, Rodrigues, & Pinheiro, 2016). While fermentation can decrease the undesirable components, it can also increase desirable components such as the total phenolic content. The increase in these phenolic compounds during lactic fermentation is associated with an increase in antioxidant activity. Which can reduce free radicals and prevent lipid oxidation (Esaki, Onozaki, & Osawa, 1994). To improve the nutritional properties of lupin, it is important to reduce components like phytate and tannin. Soaking, fermentation, or germination processes are suggested to reduce this anti-nutrient phytate. Therefore, in this study a combination of dehulling, soaking and fermentation has been used to possible reduce the phytate content along with an increase of

total phenolic content, where these processes aim to improving the nutritional profile of lupin seeds.

1.1 Objective

The aim of this thesis was to evaluate the effect of combining dehulling, soaking, and fermentation on the changes in phytate and total phenol content. And thereby improving its nutritional properties.

To achieve this aim the following objectives were set.

- Performing a fermentation with Lupin seeds and *L.Plantarum 299v* as inoculum
- Analyzing the changes in phytate and total phenols content before and after the soaking method.
- Analyzing the changes in phytate and total phenols content before, during, and after the fermentation.
- Analyzing the protein content before, during, and after the soaking/fermentation process.

2. Literature review

2.1 Lupin

2.1.1 History of the Lupin seed

Lupin is a large genus that belongs to the legume family. The exact number of different Lupin species is not known, it is estimated that it exceeds the 450. The species that are known are divided into different groups: Mediterranean, North Africa (Old World) and American (New World). The smallest group are the species that belong to the Old World, North Africa, this group only represents 12 species. To classify these 12 species the different Lupin seeds are divided into 2 groups, based on their seed coat texture. Species with a smooth coat belong to the Malacospermae group, and species with a rough coat belong to the Scabrispermae group. One of the seeds that belongs to the Malacospermae group is the *Lupinus angustifolius*, the seed used for this research (Australian government, 2013).

The growing climate of Lupin seeds varies from North America to the subtropical climate of Southern Chile and Argentina, with even some species growing in the mountains of East Africa, Iceland and Alaska. The optimum germination and growth temperature is around 20°C, while the minimum ground temperature lies around 2 to 3°C and the maximum at 30°C. Some species like the Lupinus Angustifolius can resist severe frost and can tolerate temperatures around -9°C (Australian government, 2013).

Although Lupin is not a very well-known legume when it comes to human consumption, it has been a big part of our agriculture history. Before 2000 BC, farmers in Egypt and Greece cultivated Lupin Albus for animal and human consumption. In the 1860's Lupinus Angustifolius was mostly used for green manure production in the Baltics. It was not until the 1930's till 1970's that permeable, sweet variety seeds were developed and produced on a large scale. Modifications of this Lupin species were needed for that "wild" Lupin seeds have shattering pods, high levels of alkaloids, and a hard and water impermeable coat. Which are undesirable characters when it comes to food for human & animal consumption.

What have been done in the Baltics in the 1860's is still used in Australia nowadays. Lupin like some other legumes converts atmospheric nitrogen into a more usable form that improves the quality of the soil. Farmers can use this technique when rotating between crops and thereby keeping the soil healthy.

The most Lupin that is produced nowadays is used for animal feed, mostly for animals like sheep, buffalo and cows that are able to ferment plant-based material. The reason that farmers use Lupin as animal feed is that it has a high protein and fermentable carbohydrates content combined with low levels of starch (Australian government, 2013).

2.1.2 Composition

2.1.2.1 Testa

When the testa of Lupin seed is compared to that of soy and peas it shows that Lupin has a much bigger proportion of testa than the other 2 legumes, 25% for the Lupin compared to 7 and 9% for the soy and pea respectively (Pulse, 2018).

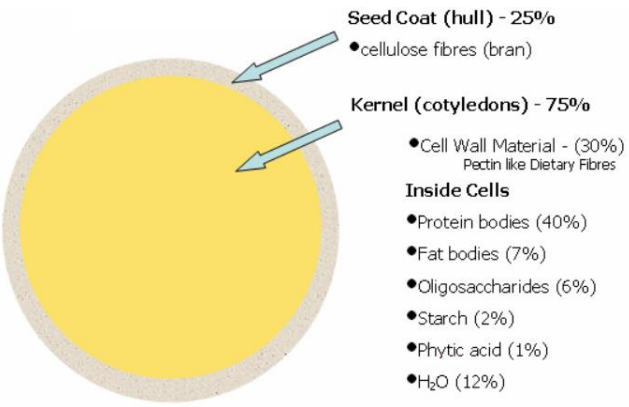

Figure 1: Schematically drawing of Lupin seed with the composition (Pulse, 2018)

In contrast to legumes like chickpeas and peas, which have a starch content that is between 50 and 70%, Lupin has an extremely low amount of starch (0.1-2%). The major health benefit of non-starch polysaccharides (NSP) like pectins, hemi-cellulose and cellulose is that they promote the growth of Lactobacilli and Bifidobacterium. The microbiota hydrolyzes these dietary fibers into monosaccharides which they ferment into short chain fatty acids (SCFA) in the anaerobic environment of the gut. Lupin seeds contain between 30-40% NSP and 6-7.6% oligosaccharides were alpha-galactoses (5.9%) and stachyose (3.8%) are the biggest group (Pulse, 2018).

Lupin contains a mixture between soluble NSP and insoluble NSP which can differ in nutritional effects. The soluble NSP form disperses when they are mixed with water and often increase the viscosity of the digesta which slows down the diffusion and improves the absorption of nutrients. Where the insoluble encloses the nutrients, making them unavailable for endogenous enzymes. In the case of Lupinus Angustifolius the insoluble NSP fraction is around 240 g/kg DM and is located for the majority in the seed coat. The seed coat consist for 50% out of insoluble NSP, where the cotyledons only contains 24% insoluble NSP (Gdala & Buraczewska., 1996). According to Gdala et al [1996] 80% of the alpha-galactose is already broken down by the small intestines before it reaches the large intestines for fermentation. When fermented, the volatile fatty acids that are produced come for 50% from these alpha-galactoses. Another effect from the consumption of NSP is that it has a high water binding capacity and therefore gives a full feeling. (Kumar, Sinha, Makkar, Boeck, & Becker, 2012). In Chile the testa of Lupin seeds are toasted and grounded to be used as dietary fiber supplements. Also in Australia the testa has been used for many years as a component in bread.

2.1.2.2 Cotyledon

The protein content in Lupin seeds varies depending of the species, from 30% with some varieties going up to 50%. This high protein content makes them a good alternative for meat, dairy and soy (Trugo, Bear, & Bear, 2003). The protein content in the *Lupinus Angustifolius* is around 36% WB, of these 36%, 56% consist out of globulins, namely α-, β-, γ-, and δ-conglutin's (Chin, et al., 2019). The most unique protein of these 4 is the γ-conglutin protein due to its controlling effect on the blood glucose levels and thus the reducing risk of type 2 diabetes

development (P. Mane, Johnson, Duranti, Pareek, & P.Utikar, 2018). Lupin has a much lower amount of inhibitor that inhibits the protein digestibility when its compared to soy <0.1 mg g^{-1} and 26 mg g^{-1} respectively. Also the high levels of lysine, leucine and threonine give Lupin proteins a higher dietary value (Bartkiene, et al., 2014). When looked at the specific proteins, 6% of the total protein fraction are the foaming proteins that consist out a combination of albumins and 2S proteins. 80% of the total protein fraction consist out of the emulsifying proteins that are a combination of α and β conglutin (Hall, Thomas, & Johnson, 2005).

Anti-nutritional properties like inhibitors of proteinase and lectins are much lower in Lupin than other legumes like soybean and peas. The alkaloid content in Lupinus Angustifolius is around 0.02%, this is much lower than other seeds that belong to the same Fabaceae family. For example *Lagiocarpus Axillaris* and *Crotalaria Retusa* that have a alkaloid content of 1,8 and 2.6% respectively (Sourakov, 2015). Also other edible seeds like corn, butterfruit and Indian-almond have a higher alkaloid content ranging from 0.2% for the corn to 3.6% for the butterfruit (Amata & Nwagu, 2013).

Lupin seeds contain a wide range of vitamins and minerals. When looked at 100 grams of *Lupin angustifolius* seeds only copper and manganese meet the daily recommended intake according to the USDA, 1.02 and 8.4 mg/100g DM resp. when 100 gram of Lupin seeds are consumed (U.S. DEPARTMENT OF AGRICULTURE, 2019). Copper along with iron, is involved in the synthesis of hemoglobin. It is also involved in the development of the skeleton, the immune system, and enzyme superoxide dismutase. So a reduced intake in copper may impair with the body's defenses. Furthermore, manganese is needed for the activation of a wide range of enzymes, including: hydrolases, kinases, transferases and decarboxylases (Anderson & Shauna, 2003). When it comes to the 3 minerals zinc, iron and calcium, the concentration varies between different cultivar. The calcium content in *Lupinus Angustifolius* is between 201-370 mg/100g DM, where the iron content is between 1.81-3.92 mg/100g DM, and the zinc content between 1.40-5.09 mg/100g DM.

The lipid content in Lupin also varies among different species. When looked at *Lupinus Angustifolius* the main fatty acids are linoleic acid (48%) followed by oleic and palmitic acid (31.2% and 7.6% resp.). According to A. Curti et al. [2018] the fatty acid composition of Lupin seeds changes when they undergo a soaking and boiling step. It is depending on the variety what changes occur, in the case of Lupin Albus the mono unsaturated fatty acids and poly unsaturated fatty acids composition increased, and so did the oleic acid, linoleic acid and linolenic acid content. A. Curti argues that this can be explained by the desaturation of internal bonds of stearic acid and the conversion to linoleic and oleic acids. In another variety the ω-6/ ω-3 ratio changed significantly (Curtia, Curti, Boninic, & N.Ramón, 2018).

Because of its water retention and fat binding capacity the kernel has been used in France and The Netherlands to increase the color and shelf-life of bread, biscuits and pasta. According to a study done at the Deakin University in Australia, replacing 10% of the wheat flour by Lupin flour significantly lowered the blood glucose and insulin levels after consumption (Hall, Thomas, & Johnson, 2005).

2.2 Phytate

Phytic acid, also knows under the chemical name myoinositol 1,2,3,4,5,6-hexakis dihydrogen phosphate (IP6) is a saturated cyclin acid that is made up of an inositol ring with six phosphate ester groups. These six inositol groups are named accordingly to their position in the molecule, IP1 to IP6. In unprocessed legumes, and cereals, IP6 is the most abundant and has therefore the strongest mineral binding capacity (FAO, IZINCG, 2018).

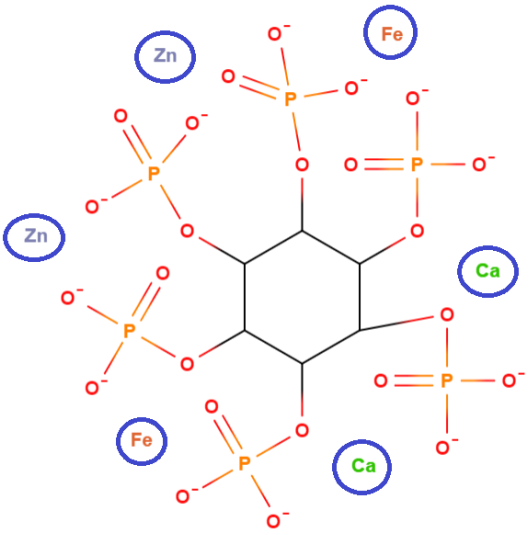

Figure 2 : Schematically drawn negatively charged phytate molecule with different cations

Phytate serves as the main storage (50-85%) of phosphorus, inositol and inorganic phosphate ions in plants. Which are used by the plant during germination for their energy metabolism. Humans are unable to metabolize phytate due to the lack of the enzyme phytase in the digestive track. Under physiological conditions (pH 6-7) phytic acid is strongly negatively charged and 8 out of 12 hydroxyl groups carry a negative charge. Because of this highly negative charge it shows a high affinity for positively charged ions like zinc, magnesium, calcium and iron. The complexes that are formed due to the gelation properties of phytic acid are called phytate which are soluble under acidic concentration like the environment of a stomach. But under conditions that are pH neutral (colon) these complexes precipitate what results in the poor bioavailability iron, zinc, magnesium and calcium. All edible plant seeds and roots contain phytate, only the concentration in the roots (+/- 0.1%) is much lower than the concentration in the seeds (+/- 75%) (Gupta, Gangoliya, & Singh, 2015). It differs from plant to plant where in the seed the majority of the phytate is located. In corn for example, 90% of the phytate is located in the kernel, where in wheat the majority is found in the outer bran layers. In Lupin seeds the majority of the phytate is restricted to a small region of cells in the cotyledons (Loewus & Tanner, 2012).

Phytate does not only bind to minerals, it also binds to proteins. According to Yueming et al. [2015], IP6 shows a strong affinity to soy proteins. Where IP5 shows some binding and the lower phosphorus forms IP1-4 almost no binding. This binding of proteins occurs mainly in acid surrounding like the stomach. Morales et al [2011] reported that the solubility of proteins reduced by 80% when sodium phytate was incubated with fish acid protease (pH 2.5). Were no reduction was reported when sodium phytate was incubated under intestinal conditions (pH 8.5). With the addition of phytase, the amino acid release was increased with 60% when incubated under acidic conditions and no increase was noticed when incubated under intestinal conditions. This research of Morales and Yueming shows that it is important to hydrolyze the phytate in the upper digestive track to improve the mineral and protein digestibility (Morales, Moyan, & L, 2011) (Dersjant, Awati, Schulze, & Partridge, 2015).

By using the anion-exchange method the phytate content in food can be determined. One of the downsides of this method is that it does not differentiate between IP6 and their hydrolysis products IP3, IP4 and IP5. By applying different processing methods such as fermentation, germination, milling, dehulling and soaking, the phytate content in legumes and grains can be decreased. Although this degradation is highly dependable on several factors such as plant species (location of the phytate), food matrix, humidity, pH and length of the processing method. The concentration of phytate varies between different Lupin species from 0.4% to 1.6%. In the variety *Lupinus Angustifolius* the phytate concentration is around 0.7% (FAO, IZINCG, 2018).

Although phytate is mostly regarded as anti-nutrient and therefore considered to have only a negative impact on the human health. Several studies have reported that phytate can prevent the formation of kidney stones, can protect against a variety of cancers and coronary heart disease. Because phytate is mostly seen as anti-nutrient the concentration is kept to a minimum. (Greiner & Konietzny, 2006).

2.3 Phytase

For the reduction of phytate, an enzyme called phytase is needed. This enzyme catalyzes the removal of phosphate from phytate and phytic acid. The removal of the phosphate goes stepwise and always starts with the fully phosphorylated phytic acids (IP6), following by IP5, IP4, IP3, IP2, and IP1. In an ideal world where the phytase would perform a complete hydrolysis, minerals, myo-inositol, and phosphate would be the end-result. However in in-vivo situations this is not the case and most of the times the end-products are a mixture of lower inositol-phosphates. This because the activity of phytase is influenced by many factors such as pH, the type of phytase, the resistance to protease, phytate content, age, and species of the animal (Dersjant, Awati, Schulze, & Partridge, 2015). The origin of phytase is not only restricted to legumes, it can also be found in cereals, fungus and bacteria. The origin of the phytase distinguish its enzyme activity. Where phytase from legume origin mostly has a phytase activity ranges from 0 to 450 FTU/Kg, grains have a much higher activity, ranging from 100 to 7000 FTU/Kg (Greiner & Konietzny, 2006) . Farmers often add phytase to the food of their animals to increase the mineral absorption. By adding 500 FTU /Kg to the food of Pangasius fish, the bioavailability of minerals increases significantly and thereby was a 3 fold increase in weight of the Pangasius measured (Debnath, et al., 2005).

2.4 Phytate molar ratios

Since 2016 the FAO, INFOODS and IZiNC compiled a global database that contains data on the phytate content of foods. The PhyFoodComp as it's called, categorized different products in different groups and gives easy access to the phytate content of more than 3500 food products (FAO, IZINCG, 2018). With the known phytate concentration, the phytate: zinc (Zn) and iron (Fe) ratios can be calculated and can predict the bioavailability of iron and zinc from food sources that are rich in phytate. According to previous research (Gibson, Smit, Vanderkooy, & Thompson, 1991) (Turnland, King, Keyes, & M. C. Michael, 1984) (Kwun &

Kwon, 1999), the bioavailability of zinc is highly dependable on 3 variables; phytate, calcium and the initial zinc concentration. Kwun et al. [1999] stated that not only phytate but also dietary calcium plays a role in the bioavailability of Zn and Fe. They reported that dietary calcium accentuate the zinc-inhibitory effect of phytate. Because of this effect of calcium on phytate the molar ratio Phy x Ca:Zn has been proposed to be more useful than Phy:Zn. Kwun stated that a molar ratio of Phy:Zn between 10-15 is considered as critical value, whereas a value above 20 is considered as a risk for clinical zinc deficiency. Furthermore, Kwun concluded that South Koreans that live in large cities have an average Phy:Zn molar ratio of 15, what indicated that they have a higher risk on zinc deficiency. Where the people that live in rural areas have a Phy:Zn ratio close to the 20. These high Phy:Zn ratios have been seen more often in other parts of Asia where the staple food consist out of grains and legumes. A molar ratio of above 200 and for Phy x Ca:Zn is considered as an important factor for zinc deficiency (Kwun & Kwon, 1999). The critical value for Phy:Ca and Phy:Fe were mentioned by Morris et al. [1985] and Hallberg et al. [1989] to be 0.24 and 1 resp. (Morris & Ellis, 1985) (Hallberg, Brune, & Rossander, 1989).

As iron deficiency is one of the most common deficiencies, phytate plays an important role in the increasing amount of people suffering from this. Phytic acid forms with iron a low soluble phytate-iron complex what inhibits the iron absorption. In regions where whole grains and legumes are the staple food this may lead to iron deficiency. This can be counteracted by the addition of proteins, beta-carotenes and ascorbic acid to the food. Ascorbic acid for example, prevents the oxidation from ferrous to the low soluble ferric form (Ems & Huecker, 2019).

2.5 Phenolic compounds

Secondary metabolites like phenols are essential for the reproduction and growth of plants. They are considered as natural antioxidants and act as protective agents during stressful conditions like oxidative stress. The interest in these phenolic compounds has increased over the last few years due to the natural antioxidant effects and risk reduction in several diseases.

When it comes to Lupin seeds these phytochemicals that contain at least 1 aromatic ring and several hydroxyl groups are divided into three main dietary phenolic compounds, tannins, flavonoids, and phenolic acids. The total phenolic content (TPC) of *Lupinus Angustifolius* is around 6 mg/g DM and between 0.9 & 2.5mg/g DM when converted to gallic acid equivalents. According to Lampart et al [2003] the majority of the total phenolic components (63%) is located in the cotyledons, and the remaining phenolic compounds (36%) are located in the seed coat (testa) (Lampart-Szczapa, et al., 2003).

Krunglevičiūtė et al [2016], found that fermenting Lupin seeds with different lactobacillus bacteria increased the total phenolic content by 5-12% depending on the strain and type of Lupin used (Krunglevičiūtė, et al., 2016). The amount of phenolic components in a plant can determine if the grain/legume/fruit is suitable for food production, further it can have a significantly effect on the sensorial properties and quality of the food. By applying a heating step before consumption the TPC can be increased due to rupture of the cells and thereby releasing the bound phenolic compounds. The content of TPC depends not only on the species

and cultivar, but also on the ripeness, location and storage condition (Nakagami, Nanaumi-Tamura, Toyomura, & K. Nakamura, 1995).

Tannins are water soluble plant phenolics that are capable of forming cross linkages with protein and other macro nutrients and thereby decreasing the nutritional value of the food. Furthermore they have been shown to decrease the bioavailability of Vitamin B12 and iron and give together with saponins a bitter taste to the seed (Lamy, Rodrigues, & Pinheiro, 2016). When it comes to tannins, they can be classified into 2 groups: hydrolysable like esters and non-hydrolysable (condensed) tannins. These hydrolysable tannins are only present in very small amounts in food product, therefore the most antinutritional effects will come from the non-hydrolysable (condensed) tannins. High levels of tannins in food products can lead to a decrease in growth rate, protein digestibility, metabolizable energy, and feed efficiency (Chung, Wong, Wei, Huang, & Lin, 2010). When looked at the tannin content in the sweet varieties, it shows that the tannin content in *Lupinus angustifolius* (0.06 –0.11%) is lower than the tannin content in the bitter variations (0.27% in *L.luteus* & 0.5% in *L.albus*) (Lampart-Szczapa, et al., 2003) (Parmdeep, Sharma, & Singh, 2017) .

According to Deshande et al [1984] the addition of 0.5-2% of tannin to the feed of poultry caused an decrease in egg production and depressed growth. When the tannin concentration reached >3% the mortality increased among the poultry test group (Deshpande, Sathe, & Salunkhe, 1984).

Phenolic acids are phenolic compounds that have only 1 carboxyl acid group. These phenolic compounds can be divided into 2 groups: hydroxybenzoic acids, including gallic acid, and into hydroxycinnamic acids including caffeic acid. When the TPC are determined the equivalents of gallic acid and caffeic acid are mostly used as measuring unit.

The capacity of phenolic compounds to inactivate free radicals is depending on the number and position of hydroxyl groups, and their aromatic rings. The double bounds of the benzene ring and the double bounds to an oxygen group in flavonoids gives them their high antioxidant activity (Minatel & Borges, 2017).

2.6 Soaking

Soaking of seeds and most legumes including Lupin is a necessary step to remove the anti-nutrients, decrease the bitterness and reduce the cooking time by 18-25% (Huma, Anjum, & Sehar, 2008). Dried Lupin absorbs water rapidly. During the first 4 hours the amount of water increases significantly, followed by an equilibrium phase, during which the Lupin approached their full soaking capacity. The capillaries on the surface of the seed coat are filled rapidly with water, this is observed as the sharp water absorption in the initial phase. During the equilibrium phase water fills the inter-micellar spaces of the lupin. After a long period of soaking (>48 hours) a small decrease will be noticed, this can be explained by the fact that water is extracted from the seed due to a concentration difference between the seed and the soaking water (Sayara & Turhan, 2001). Soaking not only decreases anti-nutrients, but also the total phenols, soluble minerals (Na, K, Mg), beta carotene, and vitamin E due to passive diffusion into the soaking water. Foods that are rich in polyphenols are often characterized by 2 major sensorial aspects, bitterness and astringency. By soaking the seeds these phenols can leach into the water and "remove" the bitterness of the Lupin.

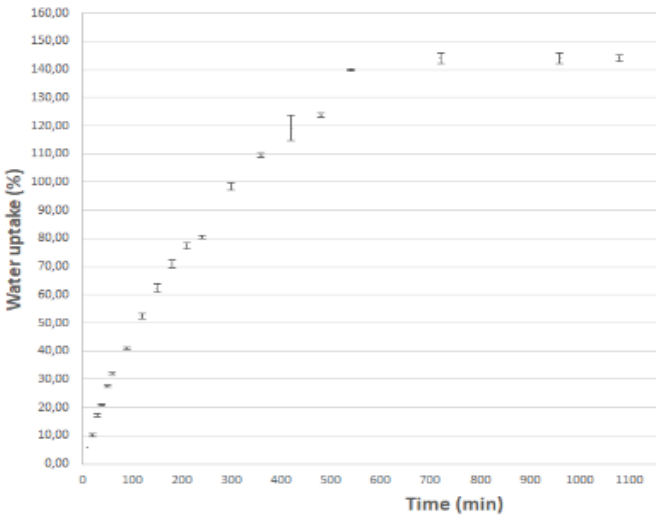

Figure 3: Absorption of water in Lupin Angustifolius over time (LUPO, 2019) .

According to Preedy et al. [2015], soaking Lupin in a solution containing 0.5% sodium carbonate reduces the phytic acid content with 16-22%, this percentage varies depending on the duration of soaking. Since phytate is very water soluble the reduction of phytic acid during soaking can be explained by the leaching of phytate in the soaking medium under influence of a concentration gradient which regulates the diffusion rate (Preedy, 2015). Not only the concentration gradient, also the pH and temperature play an important role when it comes to phytate reduction. When the pH values are between 5.0 & 6.0, and the temperature of the soaking water is between 45°C and 65°C, the most phytate is degraded. This due to the optimum temperature/pH of the phytase that is present in the seed (Greiner & Konietzny, 2006).

2.7 Fermentation

Fermentation is a simple process to preserve food that has been used since ancient times. The fermentation process has several advantages, the decreasing pH along with increasing acidity, extended shelf life, increasingly organoleptic and nutritional properties are just a few examples (Vatansever, Vegi, Garden, & Hall, 2017).

Lactic acid bacteria (LAB) are Gram-positive facultative anaerobic bacteria that are usually used in the fermentation of food products. One of the benefits of fermentation with LAB is that it improves the poor protein digestibility of most plant proteins. As a result of the partial degradation of complex proteins into more simple an soluble proteins (Krunglevičiūtė, et al., 2016). Another benefit of using LAB is that many species used for fermentation are approved by the European Food Safety Authority (EFSA) are therefore included in the Qualified Presumption of Safety list (EFSA, 2018). *Lactobacillus plantarum 299v* is a gram positive bacteria that is often used in the fermentation for its probiotic characteristics. Under favorable conditions (pH: 3-6 & temperature: 15-37°C) it produces lactic acid as the main end metabolite from fermentable carbohydrates (Soro-Yao, Brou, Amani, Thonart, & Marcelin, 2014).

Non-starch-polysaccharides (NSP) are large molecules composed of a large number of monosaccharides (>200), were oligosaccharides only consist out of a few monosaccharide units (2-6). Both of these nutrients are not hydrolyzed in the upper part of the human gastrointestinal tract due to the lack of the α-galactosidase enzyme. Instead they are fermented in the colon by the microflora. Where the major part of the dietary fibers in Lupin seeds consist out of NSP (40%), some researchers consider these NSP and oligosaccharides as anti-nutrients because their ability to reduce the feed digestibility (Kavina, et al., 2010).

The major health benefit of these components is that they promote the growth of Lactobacilli and Bifidobacterium. For this reason NSP & oligosaccharides are often used as prebiotics. The microbiota hydrolyzes these dietary fibers into monosaccharides which they ferment into short chain fatty acids (SCFA) in the anaerobic environment of the gut. However, fermentation also produces gases that generate bloating and flatulence what causes discomfort. Lupin seeds contain about 40% NSP and 13.5% oligosaccharides were alpha-galactoses (5.9%) and stachyose (3.8%) are the biggest group (Gdala & Buraczewska, 1996). Seeds and legumes contain mostly a mixture between soluble NSP and insoluble NSP. Where the soluble NSP form are mixed with water they often increase the viscosity of the digesta which slows down the diffusion and absorption of nutrients. NSP has a high water binding capacity which provides bulk to the gut and gives the consumer that full feeling. The biggest part of the NSP passes through the small intestine and only gets fermented in the colon (Kumar, Sinha, Makkar, Boeck, & Becker, 2012). According to a study done by Mulimani and Devendra [1998], legumes that undergo a soaking or cooking step will have a 33-55% and 80-87% reduction in raffinose and stachyose resp. (Mulimani & Devendra, 1998).

The proteins are also affected by fermentation, K. Lupsaite et al. [2017] found that there is a significantly increase in soluble protein content during fermentation. The soluble protein content increased with 8, 27 and 49% after a fermentation period of 24, 48 and 72 hours compared with non-fermented Lupin. These changes in soluble protein content could be

related to the proteolysis that is induced by enzymes and lactic acid bacteria during the fermentation. There are several factors like enzyme activity and acidification that could cause proteolysis. The free amino acids that are formed during proteolysis are needed by the lactic acid bacteria for their growth and acid production. While the soluble protein content increased, the total protein content decreases with 19% after 72 hours (Klupsaite, et al., 2017). Several studies have shown that the protein digestibility can be improved by fermentation (Elyas, Tinay, Yousif, & Elsheikh, 2003) (Alka, Neelam, & Shruti, 2012) (El-Hag, El-Tinay, & Yousif, 2002). The danger behind the poor digestibility of plant proteins is that it may lead to food allergies due to poor absorption in the gut. By combining soaking and cooking with fermentation the digestibility of these proteins can be increased and can even reach the same digestibility as meat proteins (Osman, 2004). By fermenting Sorghum flour with *Lactobacillus Plantarum* for a period of 36 hours, the protein digestibility increased with 92% according to Pranoto et al. [2013]. This due to the ability of Lactobacillus Plantarum to break down complex proteins and degrade tannins (Pranoto, Anggrahini, & Efendi, 2013).

The TPC in grains and legumes is fairly high, although most of these phenolic compounds are bound with other cell wall constituents such as hemicelluloses. Because of this covalently linkage the bioavailability of these phenols is limited. By applying a fermentation these bounded phenols can be converted to free phenolics, what increases the amount of higher bioactive compounds such as gallic acid and catechin. According to a study done in 2018 by Adebo et al. the flavonoid, tannin and total phenolic content in sorghum flour decreased during a 72 hour fermentation. This is possibly due to hydrolysis and degradation of these bigger phenolic molecules. While a decrease in tannins and flavonoids, a corresponding increase was noticed in gallic acid and catechin due to the breakage of the covalent bounds (Adebo, P.B, Adeyinka, & Kayitesi, 2018). While Adebo measured an decrease in TPC, other authors reported an increase in TPC (Salar, Certik, & Brezova, 2012) (Dey & Kuhad, 2014). They reported that the increase in TPC is mainly due to the metabolic activity of the microbes and β-glucosidase which are able to break down the cell wall and release free phenolics through hydrolyzation of phenolic phucosides (Oluwafemi Ayodeji Adebo, 2020).

The effectiveness of phytate degradation during fermentation is highly dependably on the phytase activity. When roasting or cooking is applied before fermentation there is a high possibility that phytase is destroyed and thereby effecting the phytate reduction. Not only the processing methods but also the amount of phytase in the raw material has influence on the degree of phytate degradation. Grains like corn, rice and oats possess a low amount of phytase, and the degradation of phytate through fermentation takes therefore a longer time (Nkhata, Ayua, Kamau, & Shingiro, 2018). According to Fritsch et al [2015]a 48-hour fermentation with *Lactobacillus Plantarum 299V* decreases the phytic acid content in Lupin flour with 13.9%. The highest reduction (24%) was noticed when Bifidobacterium Animalis BB19 was used in the fermentation of Lupin protein isolate. Although several studies establish the capacity of lactic acid to reduce phytate, others have not identified phytate-degrading enzymes produced by Lactic acid bacteria (Fritsch, Vogel, & Toelstede, 2015).

Like plant proteins have a low digestibility, plant minerals have a low bioavailability. Minerals from plants form complexes with nondigestible material such as cell wall polysaccharides and

phytates what reduces their bioavailability. Fermentation may increase the bioavailability and free minerals in flour. According to J. Suliburska et al. [2009] performing a 24-hour fermentation increases the copper content significantly from 0.58 to 0.76 mg/100g DM by releasing the copper from the plant material and phytate (Suliburska, Krejpcio, Lampart-Szczapa, & Wójciak, 2009). These minerals become available after the break down of phytate that is bounded to the minerals and thereby increases their bioavailability. However, this is counteracted by the hydrolysis of condensed tannins like proanthocyanidin to phenols. These tannins that are released will bind to the minerals what decreases their bioavailability, depending on the length of the fermentation and the initial tannin content of the legume. Another way to release the minerals is due to phytase and α-amylase that will loosen up the food matrix during fermentation what makes the minerals available. Grinding the legumes into a flour after fermentation will increase the mineral accessibility even further due to the increase grain surface and break up of cellular structure (Nkhata, Ayua, Kamau, & Shingiro, Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes, 2018).

3. Chemicals & Materials

The Folin-Ciocalteu reagents, Gallic acid, Sodium hydroxide, Acetic acid, Hydrochloric acid, Ethanol, Sodium chloride, Sodium carbonate anhydrous, FeCl3-6H2O, were acquired from VWR Chemicals (Leuven, Belgium); Sulfosalicylic dihydrate, Phytic Acid Sodium hydrate, Mixed bed resin were acquired from Sigma (Steinhem, Germany)

The Lupinus angustifolius seeds were purchased from a local farmer in Kvidinge, Sweden.

Lactobacillus plantarum 299v (Probi AB, Lund, Sweden) containing viable Lactobacillus to be used as starter culture.

4. Methodology

In this study soaking and fermentation were used to decrease the phytate and increase the total phenolic content. The preparation and handling of sample and analysis are described below. In the table below are the different processing steps shown in the different methods.

Table 1 The different processes shown in the different methods

Process steps	**Method 1**	**Method 2**	**Method 3**
Dehulling	√	√	
Soaking	√		√
Fermentation	√	√	√

4.1 Sample preparation

The Lupin seeds were sorted and any damaged seeds or impurities were removed from the batch before further processing.

The coat of the beans was manually removed, the dehulled seeds were grinded before fermentation to a flour using a laboratory mill (Bosch, Munich, Germany) and sieved with a 0.5mm sieve to obtain a fine flour. The flour was vacuum sealed and stored at 4°C until further analysis. The flour obtained in this step is only used for method 2.

4.2 Soaking

The seeds with the coat and the dehulled seeds were immerged in de-ionized water containing 0.5% sodium carbonate ($NaCO_3$) at a ratio of 1:3 (w/v) and kept at room temperature (20 °C). The beans were soaked for a period of 24 hours (Lee, 1986). After soaking the seeds were blended to a mush, and vacuum sealed until further analysis.

4.3 Fermentation

The starter culture (1%) *Lactobacillus plantarum 299v* was mixed in 1000 mL de-ionized water before usage. 500 grams of Lupin flour was mixed in a plastic container with de-ionized water containing the probiotic at the ratio of 1:2 (w/v) to a suspension. The fermentation was performed as a lactic acid fermentation.

Before the fermentation begins, 10-gram sample was taken with the aim of measuring the pH and total acidity. The suspension was then divided into two equal portions of 745 grams and kept in separate hermetically sealed fermentation tanks for duplicate. The fermentation was performed at 37°C for optimal growth of lactic bacteria. Every 3 hours, a 20-gram sample of the slurry was taken and measured directly for pH and acidity. Another sample (80 grams) was taken to be dried in the oven at 60°C until it reached a moisture content of ±3%. After the drying period the samples were vacuum sealed and stored at 4°C until further analysis (Castro-Alba, Lazarte, & Perez, 2019).

For the method with the soaking step before the fermentation, the capsule containing viable *Lactobacillus plantarum 299v* was mixed with the blended seeds. No additional water was added. The lactic fermentation follows as explained above.

4.4 Moisture content

The moisture content of the Lupin flour was determined by drying 5 gram of sample at 105 °C until constant weight (AOAC method 2000). The empty porcelain cups were weighed and 5 gram of sample was transferred to the dried porcelain cups and weighed once more. The cups containing the samples were placed in the oven at 105°C until constant weight. After 12 hours the cups were placed in a desiccator to be cooled off for a period of 30 minutes and weighed.

The loss of weight was considered as the amount of water present in the sample. Duplicated were performed (Puwastien, et al., 2011). To calculate the moisture content the following equation was used. Where M_b is the weight in grams before drying and M_a is the weight in grams after drying.

$$Moisture\ \% = \frac{M_b - M_a}{M_b} \times 100$$

4.5 pH

The pH meter was calibrated using pH 4 and pH 7 prior to analysis. To analyze the pH, 10 gram of sample were suspended in 90 mL of de-ionized water and stirred for 10 minutes. Thereafter, the suspension was filtered, and the pH of the filtered liquid was measured. Duplicated were performed (Sadler & Murphy, 2010).

4.6 Total Acidity interpreted as lactic acid

Following the method described by Sadler et al. [2010], the total titratable acidity was determined as lactic acid by weighing 10 grams of sample, mixed with 90 mL de-ionized water and stirred for 10 minutes. Of this homogenate sample, 75 mL was titrated against 0.1N Sodium Hydrochloride (NaOH) using phenolphthalein (1%) as indicator. The volume (mL) of 0.1N NaOH used was noted. The total acidity was expressed as percentage of lactic acid, the total acidity was performed in duplo. To calculate the lactic acid % the following equation was used.

$$\text{Lactic acid } \% = \frac{(\text{N x } V_t)\ x\ (100/\ M_a\ )\ x\ Eq.wt}{M_b\ x\ 1000}$$

Where N is normality of titrant (mEq/mL), V_t the volume of titrant used (mL), Eq.wt the equivalent weight of the predominant acid (mg/mEq), M_a the weight of the sample after stirring (gr), and M_b is the weight of the sample before stirring (mg).

The equivalent weight used in this equation is 90.81 mg/mEq (Sadler & Murphy, 2010).

4.7 Phytate content

4.7.1 Extraction of phytate

The phytate content was determined by using the method described by Makker et al. [2007]. 1.5 grams of Lupin flour was extracted with 50 mL of extraction solution (3.5% HCL) and mixed at 500 rpm for a period of 1 hour at 20°C. After thorough mixing, the homogenized suspension was centrifuged at 3000g for 10 minutes at 20°C. A portion (5 mL) of the supernatant was collected and diluted to 25 mL with distilled water.

To purify the phytate from the mixture, an anion exchange column (0.7 cm x 15cm) was used. The bottom was plugged with a small portion of cotton to prevent flushing of the resin. The column was vertically filled with 0.5 grams of *AGI-X8 Chloride Anion-Exchange resin* (DOWEX 1x8 Chloride form, 100-200 mesh, Sigma) to separate the current inorganic phosphorus and other components that might interfere.

10 ml of diluted sample was allowed to pass through the column. After this, 15 mL of 0.1N NaCl was transferred into the column followed by 15 mL 0.7N NaCl. This step was to first elute the inorganic phosphorus and other unwanted components, and secondly, to elute the phytate. After transferring 15 mL of 0.7N NaCl, the eluted sample was collected, the resin was discarded after one time use (Makkar & Becker, 2007).

4.7.2 Determination

3 mL of eluted sample was mixed with 1 mL of *Wade reagents* made out of 30 mg $FeCl_3x6H_2O$ and 300 mg sulfosalicylic dihydrate and vortexed for 5 seconds. The homogenous mixture was centrifuged at 3000g for 10 minutes at 20°C. The supernatant was used for phytate analysis and absorbance was read at 500nm against a blank (3mL of de-ionized water and 1mL *Wade* reagent).

A standard solution was prepared containing 1,3,5,10,20 µL/mL of phytic acid in distilled water. For this standard solution the equivalence of 100 gram of sodium phytate to 59.9 gram of phytic acid was applied. For the standard solution and blank, 1 mL of *Wade reagents* was added to 3 mL of standard solution and 3mL of blank (de-ionized water) and thereafter vortexed for 5 seconds. The homogenous mixture was centrifuged at 3000g for a period of 10 minutes at 20°C. The absorbance was measured at 500nm (Agilent 50 BIO UV, California, USA) against blank (1 mL of *Wade reagents* and 1 mL of de-ionized water) (Makkar & Becker, 2007). The phytic acid was calculated using the following equation:

$$\text{Phytic acid } (\text{g} * Kg^{-1}) = \frac{(\frac{Absorbance - Intercept}{Slope}) \text{ x Extraction x Dillution factor}}{\text{Weight of sample x } 10^3}$$

4.8 Protein analysis

Total nitrogen was determined using the Dumas system (Thermo science, Massachusetts, United States), this measures the amount of nitrogen present in the sample after complete combustion. Between 25 and 50mg was weighed in tin foil cups and used to measure the total nitrogen content. The amount of nitrogen was multiplied by a factor 6.25 to convert it to the nitrogen-protein. The gathered results were shown as crude protein in dry matter.

4.9 Total phenols

4.9.1 Sample preparation

In order to extract the bioactive compounds, 0.384 gram of Lupin flour was mixed with 9.615 mL sodium acetate buffer (0.1M, pH 5.0) to get a liquid/sample ratio of 25:1. After mixing for 10 seconds on a vortex, the samples were centrifuged in an Optima LE-80K Ultracentrifuge (Optima, Beckman, California) at 16 000 RPM for a period of 30 minutes at 4°C. The supernatant was decanted and stored until analyzing.

4.9.2 Determination of Total Phenols

For the determination of the total phenols, 0.5mL of supernatant of sample (diluted 10x) were added to a 15 mL collecting tube, where 2.5 mL of diluted (10x) Folin-Ciocalteu reagent were initially added, followed by 2 mL of saturated sodium carbonate (75 g/L). The content was mixed for 10 seconds in a vortex shaker. After mixing, the samples were heated for 30 minutes at 45°C in a water bath. After cooling down to room temperature the absorbance (Agilent 50 BIO UV, California, USA) of each sample was read at 765 nm against a blank solution containing, 2.5 mL Folin-Ciocalteu reagents, 2 mL Sodium carbonate and 0.5 mL distilled water. The standard curve containing gallic acid was used to obtain the concentration. The data was expressed as mmol gallic acid eq (GAE) / gr DM. The following formula was used to calculate the Total phenolic content. Where V is the volume used in mL, DF the dilution factor and W the weight of the sample in grams.

$$Total\ phenols\ ug/ml = \frac{\frac{(Absorbance\ +\ intercept)}{Slope}\ x\ V\ x\ DF}{W}$$

4.10 Mineral bioavailability

To determine the mineral content in the lupin the method described by (Lazarte, Carlsson, Almgren, Sandberg, & Granfeldt, 2015) was used. Firstly the material used was rinsed with a 3% nitric acid solution, followed by a double rinse with de-ionized water and thereafter washed with milk water to prevent any mineral contamination. 0.5 grams of sample was weighed and mixed in a Teflon vessel together with 3 mL HNO_3 (65%) and 2 mL H_2O_2 (30%). The Teflon vessels were closed tightly and placed in the microwave reaction system to undergo an acid digestion for a period of one hour. After this acid digestion the samples were diluted to 25 mL with milk water. Zinc and iron were determined by using the Atomic Absorption Spectrometry (AAS) with an acetylene airflow. The samples were determined at wavelengths of 213.9 and 248.3 nm respectively. A five point calibration curve was prepared with a range of 100 to 2000 mg/L for each mineral from certified AAS solutions.

The bioavailability of zinc, calcium and iron was estimated using the molar ratio between phytate and the minerals. By using the following equations the bioavailability was calculated:

$$\text{Phy: Zn} = \frac{\frac{Mg\ phytate}{660}}{\frac{Mg\ zinc}{65.4}}$$

$$\text{Phy: Fe} = \frac{\frac{Mg\ phytate}{660}}{\frac{Mg\ iron}{55.8}}$$

Where 660 is the molar weight of phytate, 65.4 represents the molar weight of zinc and 55.8 the molar weight of iron. The mineral concentration in *Lupinus Angustifolius* was gathered from the literature and not measured analytically.

5. Results & discussion

5.1 Moisture content

When looked at the moisture content of the whole seed, 3.09% ± 0.001, and the moisture content of the kernel, 3.12% ± 0.001, it can be noted that there is no significant difference (p=>0.05) between those two. According to a report of the Australian government in 2013 the whole seed has a moisture content of 9% and the kernel 12% (Australian government, 2013). These numbers can vary from the data collected during this study due to the dehydration, longer storage and possible incorrect storage of the seeds prior to analyzing. When looked at the moisture content of the flour that was obtained after fermentation, the moisture content was 2.89% ± 0.002. These values are below the threshold value, set by the WHO and FAO in 1989, for moisture content of flour should be under 14% to avoid the growth of microorganisms (FAO, 1989).

5.2 Protein content

The protein content was measured as crude protein before, during, and after the fermentation. The results of the protein content during fermentation are shown below in figure 4. As it is clearly visible in figure 4, the protein content of the seeds containing the testa have between 10 and 14% lower protein content than the seeds without the testa. This is because the testa of Lupin seeds takes up to 25% of the total weight. Thus by removing the testa, that contains mainly NSP the relative weight of the protein increases (Gdala & Buraczewska., 1996).

The protein content of the whole seed (30.8±1.388%) measured in this study is slightly lower than the protein content mentioned by other authors, who reported 32.0%. As well as the protein content in the kernel is a bit lower in this study, 39.5±0.161% compared to the 41.0% found by others (Pulse, 2018).

When looked at the different fermentations, there is clearly a difference between the 3 methods. Whereas method 3 showed a significant increase in protein content (p=<0.05). Before the fermentation the protein content was 29.4±0.305%, where during the fermentation an increase of 11% is noted to 32.9±0.144%. Method 2 started with 39.5±0.161% and showed only a significant increase (p=<0.05) of 8% to 42.9±0.540%. The seeds that were processes according to method 1 decreased from 44.0±0.644% to 42.2±0.937% but showed no significant difference when adjusted with the accuracy (±0.3%) of the equipment.

There is a mix of results mentioned by other authors, some mention a decrease in protein content where others mention an increase in protein content (Lampart-Szczapa, et al., 2006). Klupsaitea et al [2007] found no significant decrease in the protein content in the first 24 hours of the fermentation. It was after 48 hours when there was a significant decrease in protein content. They argued that the decrease in protein content during fermentation could be related to proteolysis that is caused by the natural enzymes and Lactobacillus during the

fermentation. During proteolysis, free amino acids and peptides are formed, which are necessary for the microbial growth of the Lactobacillus (Klupsaite, et al., 2017).

An increase in protein content in this study is probably due to the loss of dry matter. This loss in dry matter is caused as a result of the Lactobacillus hydrolyzing and metabolizing fats and carbohydrates for energy. Further has bacterial fermentation been known to increase the lysine content in grains what may also contribute to the increase in protein content (Nkhata, Ayua, Kamau, & Shingiro, 2018).

Table 2 Crude protein content as percentage in DM during the fermentation of method 1, 2 & 3. (n=3)

Time (h)	0	3	6	9	12	24
Method 1						
Protein %	44.01±0.444^{a}	41.22±1.148^{a}	43.32±0.710^{a}	43.58±0.202^{a}	41.39±0.544^{a}	42.22±0.937^{a}
Method 2						
Protein %	39.52±0.165^{b}	43.38±0.725^{a}	43.01±0.477^{a}	42.53±2.257^{a}	42.51±1.901^{a}	42.89±0.540^{a}
Method 3						
Protein %	29.37±0.305^{c}	30.51±0.642^{c}	31.44±0.732^{c}	33.87±0.888^{c}	33.76±1.236^{c}	32.96±0.144^{c}

Means with different superscripts in the same collum are significantly different ($p < 0.05$)Values are the average of triplicate measurements on the duplicate samples.

The protein content during and after the fermentation with the standard deviation is mentioned above in table 2.

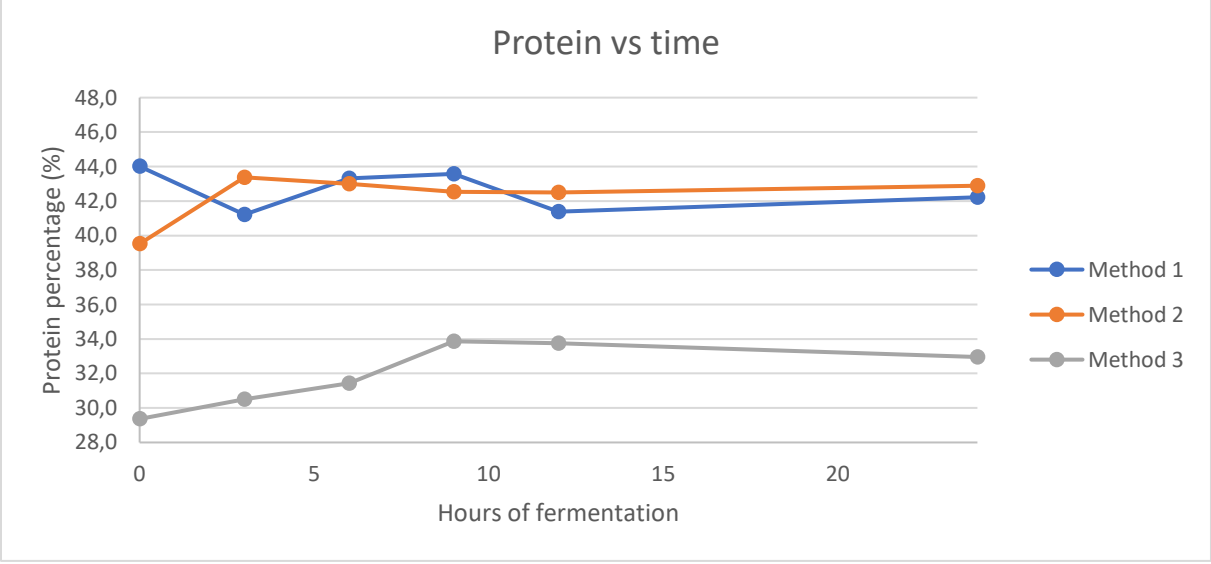

Figure 4: Crude protein content of Lupin flour presented as % protein in DM over time during fermentation.

5.3 Total acidity and pH

As is shown in the figure 5 below, the decreasing pH comes with an increasing acidity for all fermented samples. When looked at the initial pH of the three different samples, it is shown that the initial pH values are not significantly different (p=>0.05) from each other. With 6.11±0.005 for method 1, 6.02±0.020 for method 2, and 6.30±0.005 for method 3. The slightly higher pH value for method 1 & 3 can be explained by the fact that during soaking, acidic compounds such as citric or malic acid leach into the soaking water and thereby increasing the pH of the seed itself. According to Njoumi et al [2019] the pH of the soaking water deceased after a soaking period of 24 hours from 7.0 to 6.0-4.7, what argues that acid compounds leach out of the cell due to diffusion and decrease the pH of the soaking water (Njoumi, et al., 2019).

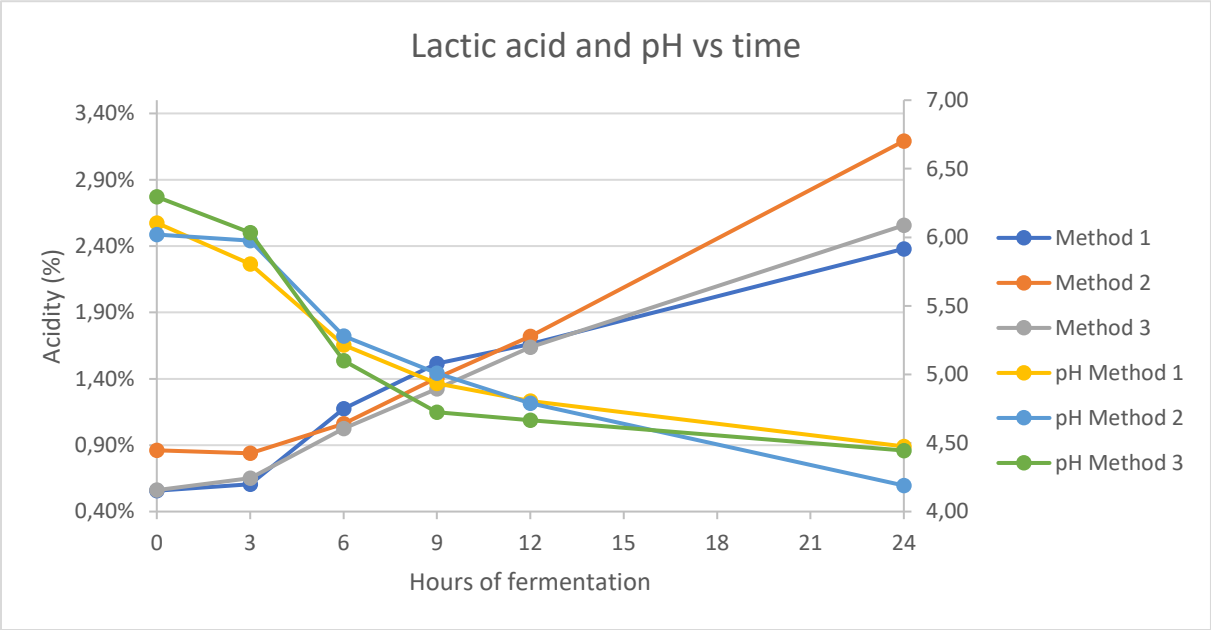

Figure 5: Total titratable acidity as percentage with pH versus fermentation time

Although other authors have mentioned lower pH values of 3.4 (Ayyash, et al., 2019) and 4.1 (Bartkiene, et al., 2014) after a 24 hour fermentation. The higher observed pH in this study can be due to the not well degradable oligosaccharides of this Lupin variety. Where addition of maltodextrin could possible reduce the pH.

With an initial lactic acid percentage (LA%) of 0.557%±0.000 for method 1, 0.862%±0.000 for method 2, and 0.561%±0.000 for method 3 there is a clear significant difference between the soaked seeds and the seeds that were not soaked (p=<0.05). This lower LA% of the soaked seeds can be explained as a result of leaching of acid compounds in the soaking water, and thereby reducing the amount of acids present during the start of the fermentation. Considering that the soaking water was completely absorbed by the seeds and not decanted prior to the fermentation this is highly unlikely. All the acid compounds that might have leached out of the seeds were still present during the fermentation.

When the LA% results gathered in this study are compared with the LA% results of different studies it shows almost no difference. With LA% contents ranging from 2.3-2.7% after a 24

hour fermentation Bertkiene et al. found similar values as this study (Bartkiene, et al., 2014). Although these values cannot be compared one on one due to the fact that Bartkiene et al. used different Lupin species and different Lactobacillus than this study. Further did Bartkiene et al. not mentioned the initial LA% of the Lupin seeds, and therefore it is not possible to compare the increase of LA% during the fermentation.

It is important to have a solid increase of TTA and decrease of pH in any product that undergoes a fermentation. Where the low pH and high acidity increase the shelf life and can decrease the risk of microbiological contamination, it also shows the lactobacillus activity. In table 3 are the results of these fermentations noted, it is clearly visible that the LA% of the seeds that underwent a 24-hour soaking period is lower than the seeds that did not were subjected to a 24-hour soaking period.

Table 3: pH and total titratable acidity presented as % Lactic acid (LA%) of method 1, 2 & 3 during 24 hour fermentation. Results are shown every 3 hours until 12 hours, afterwards only 24 hour after initial start of fermentation.

	0	3	6	9	12	24
Method 1						
pH	6.11±0.005	5.81±0.005	5.22±0.035	4.92±0.055	4.81±0.005	4.48±0.005
LA%	0.557±0.000^{a}	0.607±0.000^{a}	1.175±0.000^{a}	1.616±0.000^{a}	1.662±0.001^{a}	2.380±0.002^{a}
Method 2						
pH	6.02±0.020	5.98±0.025	5.28±0.030	5.01±0.010	4.79±0.010	4.19±0.010
LA%	0.862±0.000^{b}	0.839±0.001^{b}	1.062±0.000^{b}	1.407±0.000^{a,c}	1.720±0.001^{a}	3.192±0.000^{b}
Method 3						
pH	6.30±0.005	6.04±0.035	5.10±0.050	4.73±0.025	4.67±0.015	4.45±0.045
LA%	0.561±0.000^{a}	0.652±0.001^{a}	1.026±0.000^{a,b}	1.325±0.000^{c}	1.639±0.000^{a}	2.557±0.000^{a}

Means with different superscripts in the same collum are significantly different ($p < 0.05$)Values are the average of duplicate measurements

5.4 Phytate content

Several authors mention an phytate concentration of 0.4-1.6% depending on the species and cultivar (Trugo, Donangelo, Duarte, & C.L.Tavares, 1993) (Parmdeep, Sharma, & Singh, 2017). The species used in this study has according to the FAO a phytate concentration around 0.7% in the whole seed (FAO, IZINCG, 2018), which is lower than the 1.0±0.294% found in this study. Where according to Loewes and Tanner the majority of the phytate is located in the cotyledons, this study found an increase of 22.4% from 1.041±0.294% to 1.275±0.017% after dehulling the Lupin seeds. What indicates that removing the testa, which contributes to 25% of the total weight, causes the relative increase in phytate content. And thus that the majority of the phytate is located in the cotyledons (Loewus & Tanner, 2012). Where a 24-hour soaking period of whole Lupin seeds reduced the phytate content with 2.5% from 1.041±0.294% to 1.032±0.500%. A combination of dehulling and a 24-hour soaking period shows to be more affective and significantly ($p=<0.05$) reduced the phytate content with 19% from 1.041±0.294% to 0.857±0.024%. The results gathered in this study are in line with other studies. According to Embaby [2010] dehulling *Lupin albus* increased the phytate content with

14%, and a 24-hour soaking period decreased the phytate content in whole *Lupin albus* seeds with 3.0% (Embaby, 2010)

During this study it was expected to see a decrease in phytate content after a 24-hour soaking period and a 24-hour fermentation. However, the small decrease in this study after a 24-hour soaking period was not significant, neither was the 3.0% decreased mentioned by Embaby [2010] in their study (Embaby, 2010).

At the end of the fermentation of method 1 there was a small decrease noted of 5.8% from 0.857±0.024 g/kg to 0.807±0.110 g/kg. Although after 24 hours there was a small decrease, during the fermentation there was a significant increase in the phytate content (p=<0.05). After a fermentation period of 12 hours there was an increase of 55% in phytate content measured, this increase can be due to inefficient separation of the used resin and thereby not separating the phosphates and phytate what can explain an increase in phytate content. Method 2 showed after a 24-hour fermentation a small not significant (p=>0.05) decrease of 2.2% from 1.275±0.017g/kg to 1.248±0.111 g/kg. The seeds that were processed according to method 3 showed a slightly larger yet not significant (p=>0.05) decrease of 11% after the 24 hour fermentation period from 1.032±0.500 g/kg to 0.919±0.348 g/kg.

There can be several reasons for this fluctuation, firstly the low quantity of enzyme phytase in this specific seed variety, and secondly the deactivation of the enzymes present in the seeds and thereby reducing its enzyme activity (Greiner R. , 2002). Fritsch et al [2015] conducted a study where they incubated different microbes for a period of 48 hours in MRS broth containing 0.2% phytic acid. Their goal was to determine if the selected strains were able to metabolize phytic acid. One of their selected strains was *Lactobacillus plantarum*, which showed with 54% the highest decrease in phytic acid. Although this was a model experiment conducted in MRS broth agar, it shows that the strain used in this study is able to reduce the phytic acid content, however, the reduction also depends on other factors such enzyme concentration, temperature and pH for enzyme activation. When Fritsch et al. performed a 48-hour fermentation under optimal conditions according to the different bacteria on flour of *Lupinus angustifolius boregine*, the fermentation with *Lactobaillus plantarum* showed a decrease of 13.9% in phytic acid. Where other bacterial strains such as *Pediococcus pentosaceus, Lactobacillus lactoc,* and *Bifidobacterium animalis* showed a decrease of 0.7, 6.9 and 0.0% respectively in phytic acid. Although this argues that *Lactobacillus plantarum* has the ability to decrease phytic acid in Lupin flour, the same experiment was done with a different Lupinus cultivar: *Lupinus angustifolius azuro*. Where in this experiment *Lactobacillus plantarum, Pediococcus pentosaceus, Lactobacillus lactoc,* and *Bifidobacterium animalis* showed a decrease of 14, 9.3, 0.0 and 0.0% respectively. The higher decrease (from 0.7 to 9.3%) in phytic acid degradation with *Pediococcus pentosaceus* and the lower decrease (from 6.9 to 0.0%) with *Lactobacillus Lactis* can suggest that the cultivar that is used for this study is possible not greatly affected by *Lactobacillus plantarum 299v* (Fritsch, Vogel, & Toelstede, 2015). Because the cultivar that is used in this study is not known it is hard to tell if the phytase

activity is decreased due to wrong storage or that the Lactobacillus species has no influence on this cultivar

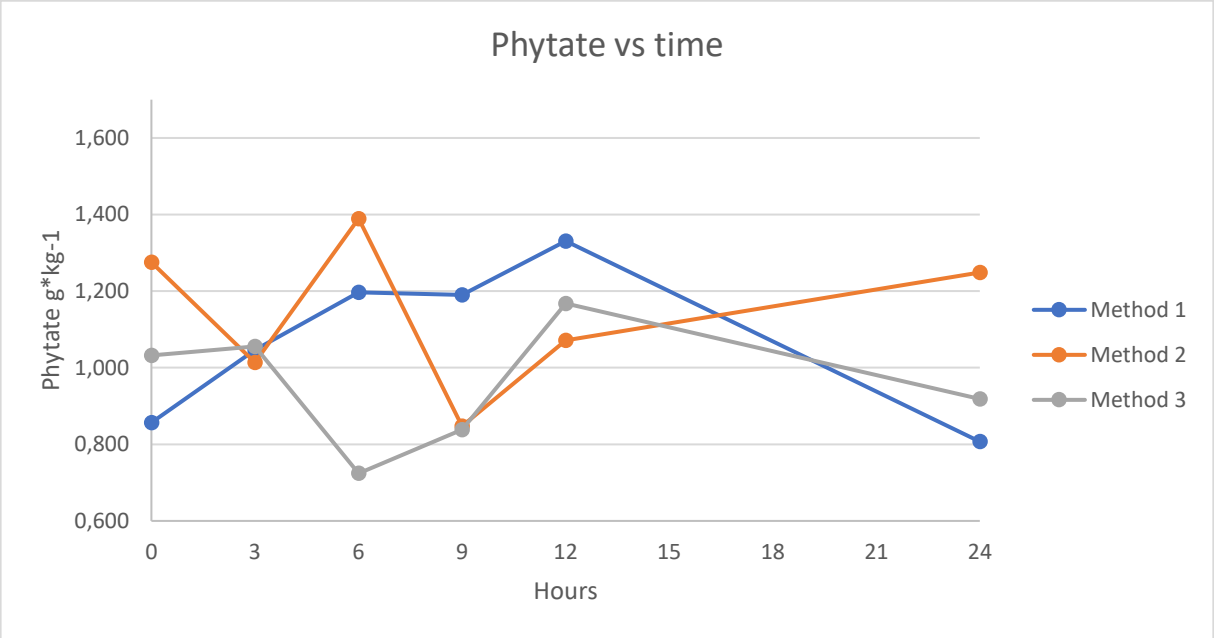

Figure 6: The phytate content over time during fermentation with Lactobacillus Plantarum 299v

When it comes to the phytase from legumes and especially Lupin species there is not much research done. Greinier et al. [2002] was one of the first researchers that tried to purify phytase from *Lupin albus amiga*. From their study they concluded that the phytase activity increased from 0.017 FTU /g DM to 0.097 FTU/ g DM during a 4-day germination period in a dark room of 20°C. After 4 days the phytase activity started to decrease. The pH range for Lupin phytase is 3.5 to 8.0, with an optimum activity at 5.0. The optimum pH of 5.0 was found at a temperature of 50 °C (Greiner R. , 2002). The difference in optimum temperature of the phytase and the temperature used during the fermentation and soaking can be a reason why there was no significant reduction of phytate. It can also be noted that the phytase content in Lupin (0.017 FTU/g) is 40x lower than the phytase content in wheat (0.700 FTU/g) (Drakakaki, 2005). What can contribute to the low enzyme activity and phytate reduction in Lupin seeds.

Another reason for the low phytate reduction can be the conditions used during soaking. The temperature and pH of the soaking water are essential. When the temperature of the soaking water is kept between 45-65 °C and the pH between 5.0 & 6.0, which are close to the optimum conditions for phytate dephosphorylation, more phytate can be enzymatically hydrolyzed (Greiner & Konietzny, 2006).

5.5 Mineral molar ratio

Unfortunately, it was not possible to analyze the mineral content of the Lupin flour before and after the fermentation due to the outbreak of COVID-19. The concentration of zinc, iron and calcium used in the calculations were therefore obtained from other literature. According to Trugo et al. [1993], *Lupinus angustifolius* flour contains between 4.8-5.5 mg/100g zinc and 3.2-5.3 mg/100g iron depending on the cultivar (Trugo, Donangelo, Duarte, & C.L.Tavares, 1993). The average calculated Phy:Zn and Phy:Fe ratios of Lupin flour using the phytate concentration from this study are 20.3 and 22.3 respectively. Both for the Phy:Zn and Phy:Fe ratio are much higher than the critical value of 10 and 1 respectively reported by Hallberg et al [1989] (Hallberg, Brune, & Rossander, 1989). The Phy:Zn molar ratios found in this study are however in the same range as the ones found by Trugo et al. (Phy:Zn; 15.0-23.2). Where this study found a phytate concentration of 1.0 g/100g, Trugo et al. found phytate levels ranging between 0.7-1.1 g/100g, with more cultivars having a phytate concentration of 0.7 and 0.8 g/100g. When the Phy:Zn ratios of the *Lupinus angustifolius* are compared to the molar ratios of other legumes with similar zinc concentrations, it is shown that the initial phytate concentration determines the outcome. According to Ferguson et al [1988] kidney beans have a zinc and phytate concentration of 3.2 mg/100g and 1.2 g/100g respectively with a Phy:Zn ratio of 35. Where lima beans have a zinc and phytate concentration of 4.4 mg/100g and 0.7 g/100g respectively with a Phy:Zn ratio of 16 (Ferguson, Gibson, Thompson, & Ounpuu, 1988). Although the results calculated in this study do not represent the reality, it is highly possible that these molar ratios are indeed way above the critical value.

5.6 Total phenol content

The total phenolic content increased during the fermentation in all of the different methods, In table 4 are the results shown of the total phenolic content during the fermentation. With at the start of the fermentation, 0.85 ±0.274 mg GEA/g for method 1, 1.37 ±0.101 mg GAE/g for method 2, and 1.10 ±0.274 mg GAE/g for method 3.

Table 4: Total phenolic content of Soaked, not soaked, and soaked + coat during a 24 hour fermentation, where TPC: Total phenolic content presented as mg GAE/g. (n=2)

	Time of fermentation (hours)					
	0	3	6	9	12	24
Method1						
TPC	0.85 ±0.045^{a}	1.60 ±0.163^{a}	2.05 ±0.173^{a}	2.33 ±0.112^{a}	2.33 ±0.253^{a}	3.51 ±0.348^{a}
Method 2						
TPC	1.37 ±0.178^{b}	3.26 ±0.187^{b}	3.21 ±0.262^{b}	3.88 ±0.213^{b}	4.46 ±0.326^{b}	6.62 ±0.290^{b}
Method 3						
TPC	1.10 ±0.143^{a,b}	1.42 ±0.352^{a}	2.04 ±0.181^{a}	3.32 ±0.175^{c}	4.41 ±0.663^{b}	4.80 ±0.147^{c}

Means with different superscripts in the same collum are significantly different ($p < 0.05$)Values are the average of duplicate measurements on the duplicate samples.

Method 1 showed a significant lower TPC at the start of the fermentation (time 0) compared with method 2. This can be explained by the leaching of the water soluble phytochemicals in the soaking water. Results found in this study are comparable to the results found by other authors. The TPC in the whole seed found in this study (1.3 ±0.005 mg GAE/g) is in the same range as the 1.1, 1.6 and 1.8 mg GAE/g found by others (Oomah, Tiger, Olson, & Balasubramanian, 2006) (Lampart-Szczapa, et al., 2003) (Czubinski, Dwiecki, Siger, & al., 2012).

With an 3.3-fold increase in TPC to 3.51 ±0.348 mg GAE/g for method 1, a 3.8-fold increase to 6.62 ±0.290 mg GAE/g for method 2, and 3.4-fold increase to 4.80 ±0.147 mg GAE/g for method 3 there is a clear difference between the TPC after a 24 hour fermentation. The increase in total phenols during fermentation is something other authors have mentioned as well. Vita et al [2016] mentioned an increase of 8% during a 24 hour fermentation. Although the increase in this study is much higher as mentioned by Vita et al. this could be explained by the different bacterial strain and lupin variety used in their study, which makes it impossible to compare the results one on one (Vita Krunglevičiūtė, 2016). Where other studies have mentioned similar results, Khan et al [2018] mentioned that after a 24-hour fermentation the TPC content in *Lupinus angustifolius* increases between 300-450% (Khan, 2018).

A similar trend was noticed in other research, a study by Curiel et al. [2015] showed that after a 24 hour fermentation with *Lactobacillus plantarum* the TPC increased with 20-70% along with a 10% decrease in condensed tannins (Curiel, et al., 2015). Although the fermentation was performed in other legumes (chickpea, pea, and kidney bean) multiple papers have shown that fermenting legumes with LAB increases the TPC and decreases the condensed tannins (Coda, et al., 2015) (Pasquale, Pontonio, Gobbett., & Rizzello, 2019). *Lactobacillus plantarum* produces an enzyme called tannase which is responsible for the hydrolysis of ester bonds in tannins (Rodríguez, et al., 2009). The importance of reducing tannins in legumes is because tannins are considered as anti-nutrients and have the ability to decrease the protein digestibility, damage to the mucosal lining, reduction in hemoglobin and increase excretion of essential amino acids, they also chelate divalent minerals and reduce their bioavailability. In addition, tannins alongside saponins are compounds that give the Lupin seed its bitter-taste (Lamy E. , 2016).

As is shown in this study and mentioned by others is that fermentation has a positive influence on the TPC in Lupin. Therefore the increase in TPC all depends on the microorganism species that is used for the fermentation. *Lactobacillus plantarum* is one of the LAB that is able to produce β-glucosidase what is capable to hydrolyze several flavanols and isoflavone glycosides. Further, has the presence of LAB during the fermentation influence on the conversion from bigger phenolic compounds to smaller phenolic compounds, alongside with the depolymerization of high molecular weight molecules (Othman, Roblain, Chammen, Thonart, & Hamdi, 2009). When looked at other legumes such as cowpeas, 30% of the total phenolics are insoluble bound phenolics. These 30% are covalently bound to components in the cell wall like lignin, hemicellulose, and cellulose. The phenolic acids that are present in these legumes forms ether linkages with these cell wall components through their hydroxyl groups, and ester linkages with carbohydrates and proteins. During the fermentation,

microorganisms such as *Lactobacillus plantarum* are able to break those ester links and thereby releasing the bound phenolics (Estrada, Uribe, & Saldívar, 2014).

It is already mentioned by other authors that there is strong evidence that suggest that reactive oxygen species (ROS) can lead to damage in protein, DNA and lipid structures (Wang & Clements, 2008). Antioxidants prevent auto-oxidation of food and act as free radical terminators what degrades these ROS and free radicals to non-reactive forms. The majority of these antioxidants are polyphenolic compounds and thus by performing a fermentation the levels of antioxidants can be increased. Starkute et al. [2016] reported an increase of 60-88% in antioxidant activity after a 48-hour fermentation of *Lupinus angustifolius* with *Lactobacillus sakei* (Starkute, et al., 2016). In 1994 Esaki et al. suggested that the increase in antioxidant activity was possible due to ''the liberation of lipophilic aglycones by catalytic action of β-glucosidase during fermentation'', where the bacteria used in this study, *Lactobacillus plantarum,* is one of the LAB that produces this enzyme (Esaki, Onozaki, & Osawa, 1994).

6 Conclusion

From the results gathered in this study, it can be concluded that the phytate content is significantly reduced by 19% when a combination of dehulling and soaking is applied. Where a 24-hour fermentation also reduced the phytate content, these results were not significant. Performing a 24-hour fermentation does however show an 3-fold increase in total phenolic content.

7 Further recommendations.

To improve the reduction in phytate, the seeds can be soaking at 45-50 °C and pH of 5.0-6.0 to promote the activation of phytase. Also drying the seeds after a 24-hour soaking period for 12 hours at 50 °C might improve the phytase activity. The phytase activity can be measured to check if there is even any phytase available in the seeds. The tannin content can be measured during the fermentation, where several authors have mentioned a decrease in tannins during their fermentation process. It is also possible to conduct a sensorial test on the Lupin flour containing the testa, where it is very labor intensive to remove the testa from the kernel.

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