Fermentation of lupin with lactic acid bacteria using a bioreactor

Master's Thesis in Technology
University of Turku
Department of Life Technologies
Master's Degree Programme in Food Development
February 2024

Malviina Nikola

UNIVERSITY OF TURKU
Department of Life Technologies
NIKOLA, MALVIINA: Fermentation of lupin with lactic acid bacteria using a bioreactor
Master's Thesis in Technology, 36 pages.
Food Development
February 2024
The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

The rising interest in incorporating lupin into our diet is driven by its unique nutritional value, notably its high protein content, coupled with its sustainability. Fermentation is a natural bioprocess widely employed to enhance shelf life and microbiological safety. Lactic acid bacteria fermentation, that involves the production of lactic acid from glucose, has shown promise for usage in plant-based foods. The aim of this project is to examine the effect of lactic acid bacteria fermentation on dairy analogue made from lupin flour.

Lupin flour was mixed with tap water using a blender and liquid fraction was separated from solids by centrifugation. Fermentation was tested with three *Lactiplantibacillus plantarum* strains for 72 h at 30 °C. Final fermentations using a bioreactor was done for 24 h at 28 °C. Samples were taken out at the different timepoints, and pH was measured through fermentation process. Simple sugars and organic acids were analysed quantitively using gas chromatography with flame ionization detector.

The results indicated the success of the fermentation process, with sugars being converted into lactic acid. Noteworthy findings revealed no significant differences among the employed bacterial strains, except for distinct effects observed in citric acid levels.

Keywords: bioreactor, fermentation, GC-FID, lactic acid bacteria, lupin

Content

Abbreviations

GC-FID	Gas Cromatography with Flame Ionization Detection
HDL-C	High-density lipoprotein cholesterol
LAB	Lactic acid bacteria
LDL-C	Low-density lipoprotein cholesterol

1 Introduction

1.1 Plant-based diet

Population growth, competition for natural resources, climate change, and other similar issues in modern world are threatening the current agriculture and food systems. That leads to the need of more sustainable food products and food production systems. United Nations Member States have promised to find solutions to the aforementioned problems. (Alcorta et al. 2021.)

Plant-based diet focuses on foods primarily from plants. The diet has gained popularity as more people start to follow it every year. Plant-based dairy alternatives' market has expanded worldwide. Sales of dairy alternatives have doubled from 2009 to 2015 and the popularity continues to grow each year (Figure 1). This can be explained by changes in consumer lifestyle, desire for clean-label, allergies, and balanced way of eating. Additionally, possible health benefits of plant-based dairy alternatives attracts consumers. (Alcorta et al. 2021; Pritulska et al. 2021; Sridhar et al. 2022) Dairy alternatives have to overcome some sensory challenges among people who consume regular cow´s milk. Plant-based alternatives however are often perceived as a more sustainable choice which attracts some consumers to change dairy products to plant-based alternatives.(Alcorta et al. 2021) Plant-based yogurt alternatives are seen as healthy and natural by consumers but the low protein content and less appealing texture drive away some consumers. (Boeck et al. 2021)

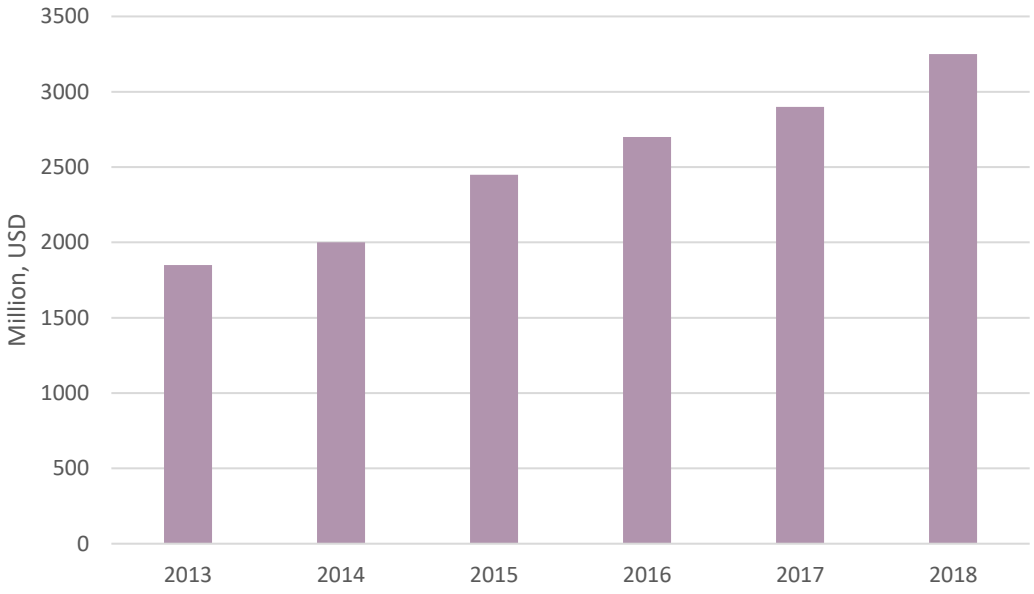

Figure 1: Sales of plant-based dairy milk alternatives by million USD *(Pritulska et al. 2021)*

1.1.1 Environmental benefits of plant-based diet

Meat and dairy alternatives and other plant-based products are usually presented as less harmful to environment and more sustainable. Plant-based diet have shown to reduce the effect of global warming and environmental pollution. However, the sustainability of plant-based products is still a subject to study. There is clear evidence that legumes have low environmental footprint and animal-based diet contributes negatively to environment. (Alcorta et al. 2021; Detzel et al. 2022) The real influence of legumes in reducing greenhouse gas emissions depends a lot on the agro-ecosystem used and on the post-harvest processing (Alcorta et al. 2021). On the other hand, livestock farming has a large environmental footprint. Around 10 % of the European Union's (EU) greenhouse gas emissions are caused by agriculture and almost 70 % of those are from meat production. Second largest source of greenhouse gas emissions in agriculture is dairy products. In addition to greenhouse gases, livestock farming causes nitrous oxide and ammonia emissions as well as nitrogen and phosphorus pollution. (Detzel et al. 2022) Generally, milk production has a bigger environmental impact than plant-based alternatives except for almond milk that has a considerably bigger impact due to its production methods and transportation (Alcorta et al. 2021). On top of these aforementioned environmental benefits, plant-based diet can be beneficial in other ways as well. For example, plant-based diet also increases peoples willingness to contribute to animal welfare organizations (Fehér et al. 2020).

1.1.2 Health benefits of plant-based diet

Plant-based diet focuses on consumption of foods primarily derived from plants, such as fruits, vegetables, nuts, and legumes. The diet may include small quantities of food from animal origin. People that follow plant-based diet might substitute animal products to plant alternatives without permanent exclusion of animal foods. (Alcorta et al. 2021)

Plant-based diet has been proven to have some health benefits such as preventing many diseases. Additionally, food processing and preparation techniques can improve the functionality of the final plant-based food products even more. (Sridhar et al. 2022) People that follow a plant-based diet often have higher quantity of important nutrients such as magnesium, potassium, and antioxidants due to their diverse diet (Fehér et al. 2020).

Plant-based diets have beneficial effects of mostly reducing the risk of obesity and diabetes by reducing body fat and decreasing the intake of saturated fat. That can be a result of the diet including foods rich in fibre, antioxidant content, magnesium, and phytochemicals, which have been shown to increase insulin sensitivity and glycaemic control. (Alcorta et al. 2021.)

Plant-based diet is observed to be cardioprotective and reducing the risk of developing diseases such as dementia, gallstones, kidney diseases, rheumatoid

arthritis, and some allergies and also reduces the likelihood of developing cancer. The diet may be helpful for both, the prevention and the treatment of, some conditions such as high blood pressure. Some conflicting results have been found about the impact of plant-based diet on high density lipoprotein-cholesterol (HDL-C) and low density lipoprotein-cholesterol (LDL-C). For instance, some studies show on difference only in plasma HDL-C levels between vegetarians and omnivorous diets but other studies have shown that vegetarian diet to lower total cholesterol and both LDL-C and HDL-C.(Fehér et al. 2020; Alcorta et al. 2021)

1.1.3 Proteins in plant-based diet

In the European Union, the majority of protein consumption consists of animal-based sources such as meat and dairy products, comprising over 50% of the overall protein intake. Notably, a significant portion of pulses and cereals consumed in the EU is allocated for animal feeding.(Detzel et al. 2022) In Finland, diets are notably protein-rich, with less than one-third of protein intake coming from plant-based foods. The primary sources of protein are meat for men and meat and dairy for women (Figure 2), while pulses, nuts, and seeds contribute to less than 5% of the overall protein intake.(Valsta et al. 2018)

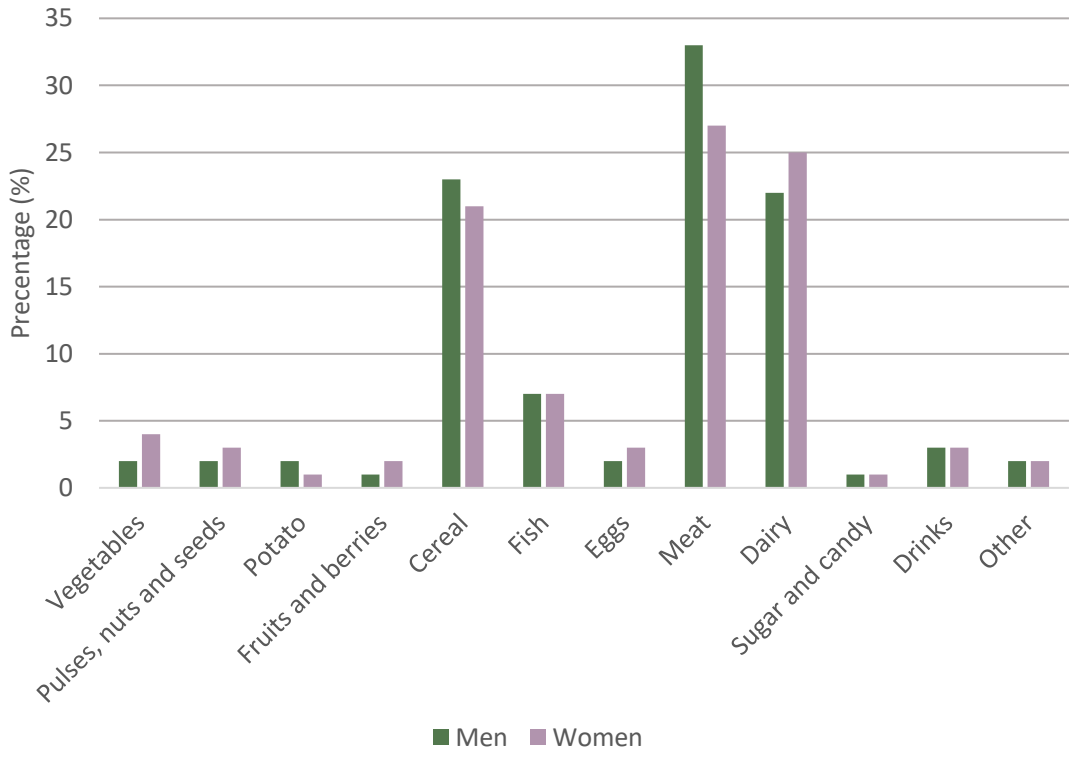

Figure 2. Percentage of protein intake by category in Finland. Source: *Valsta et al. 2018)*

Criticism of plant-based diets often centres on their perceived low protein content and unfavourable amino acid composition. The risk associated with low protein intake has been even considered a barrier to transitioning to a plant-based diet.

However, numerous studies suggest that there is no significant difference in protein supply between plant-based diets and those incorporating animal products. In fact, nowadays, a variety of protein-rich plant-based alternatives are readily available. Cereals and tree nuts naturally contain less protein than legumes and are used less frequently due to their higher costs. Pulses, on the other hand, boast a higher protein content ranging from 20% to 36 % and soybeans often match the protein content of bovine milk products.(Fehér et al. 2020; Alcorta et al. 2021; Boeck et al. 2021.)

Proteins play a crucial role in maintaining a properly functioning body and promoting overall health by forming tissues and acting as regulators. The nutritional quality of food proteins is determined by their ability to meet essential amino acid requirements for growth and tissue maintenance. Amino acid content and protein structure, influenced by processing conditions and interactions with other ingredients, are pivotal aspects of dietary proteins. Protein modification through fermentation, which leads to the degradation of plant cell walls and the release of antioxidants, is a practical method for increasing the presence of bioactive chemicals.(Bartkiene et al. 2018; Alcorta et al. 2021.)

The debate surrounding the quality of plant protein has intensified with the growing interest in plant-based diets. While some plant proteins exhibit a similar amino acid profile and bioavailability as animal proteins, anti-nutrients in plant-based foods can impact protein absorption, although most food processing technologies mitigate these effects. Studies indicate that vegetarians often have lower protein consumption, particularly of lysine and methionine amino acids, due to the lower concentrations of these amino acids in plant proteins. However, a well-planned and balanced plant-based diet does not result in protein deficiency.(Alcorta et al. 2021.)

Developing desirable plant-based dairy alternatives presents a challenge in achieving both acceptable sensory experiences for consumers and matching the nutritional value of traditional dairy products. Nutritional value is sometimes bolstered through fortification with vitamins and amino acids, while desirable sensory experiences can be achieved through fermentation or the addition of specific ingredients. Bulking agents like maltodextrin, fibres such as inulin, or thickeners like gellant may be added to mimic the mouthfeel or structure of dairy products.(Boeck et al. 2021.) Fermentation not only diminishes beany flavours but also imparts desirable volatile flavours. Plant-based yogurts are created through fermentation, which also enhances the solubility and amino acid composition of plant proteins. Studies reveal that fermenting soybeans with *Lactiplantibacillus plantarum* increases the essential amino acid content. (Alcorta et al. 2021.)

1.2 Plant-based milk alternatives

The rise of vegetarianism, veganism, and similar dietary preferences has led to a surge in the popularity of plant-based milk alternatives. These alternatives are also needed by consumers with cow's milk allergies. Furthermore, plant-based milk alternatives serve as crucial components in many vegan dairy substitutes, including cheese, yogurt, and ice cream. Health-conscious consumers are also increasingly opting for plant-based milk alternatives, and plant-based dairy alternatives are often perceived as a more sustainable choice. (Aydar et al. 2020; Alcorta et al. 2021.)

There is often an aspiration for plant-based milk alternatives to mimic the technical, nutritional, and organoleptic properties of cow's milk The taste of plant-based ingredient is the greatest factor influencing consumers adaption to dairy alternatives. (Alcorta et al. 2021.) Typically, these plant-based drinks are created by crushing plant materials and extracting soluble components into water. The ultimate physicochemical characteristics depend on the raw materials and the processes employed. Various techniques are applied to enhance the homogenization and stability of plant-based milks, making them more akin to animal milk, which is a natural emulsion. Plant-based drinks made from cereals, pseudo-cereals, or other starchy materials may easily gel during sterilization (Figure 3). Raw materials with excessive lipid content, such as seeds and nuts, can lead to phase separation and diminished product stability. (Tangyu et al. 2019.)

Some raw materials used in plant-based milk alternatives have a protein content similar to cow's milk. More importantly, plants often offer specific nutritional properties, being rich in micronutrients and containing bioactive compounds (Figure 3). These contribute to the health benefits associated with a plant-based diet. Nuts, for instance, contain significant amounts of vitamins E and C, known for their antioxidant properties. Legumes serve as a good source of essential mono- and polyunsaturated fatty acids, minerals, and phytoestrogens. In certain plant materials, β-glucans contribute to health effects by lowering cholesterol levels and enhancing sensory attributes through added thickness. However, the protein content of most plant-based milk alternatives is low or non-existent. Plant proteins often exhibit limitations in essential amino acids, with low levels of L-Lysine, L-methionine, L-cysteine, and L-tryptophan. Additionally, vitamins D and B12 are typically almost absent in plant-based milk alternatives. Plant-based oligosaccharides, such as raffinose, stachyose, and verbascose, can only be digested by intestinal bacteria through fermentation, potentially leading to flatulence or diarrhoea. (Tangyu et al. 2019.)

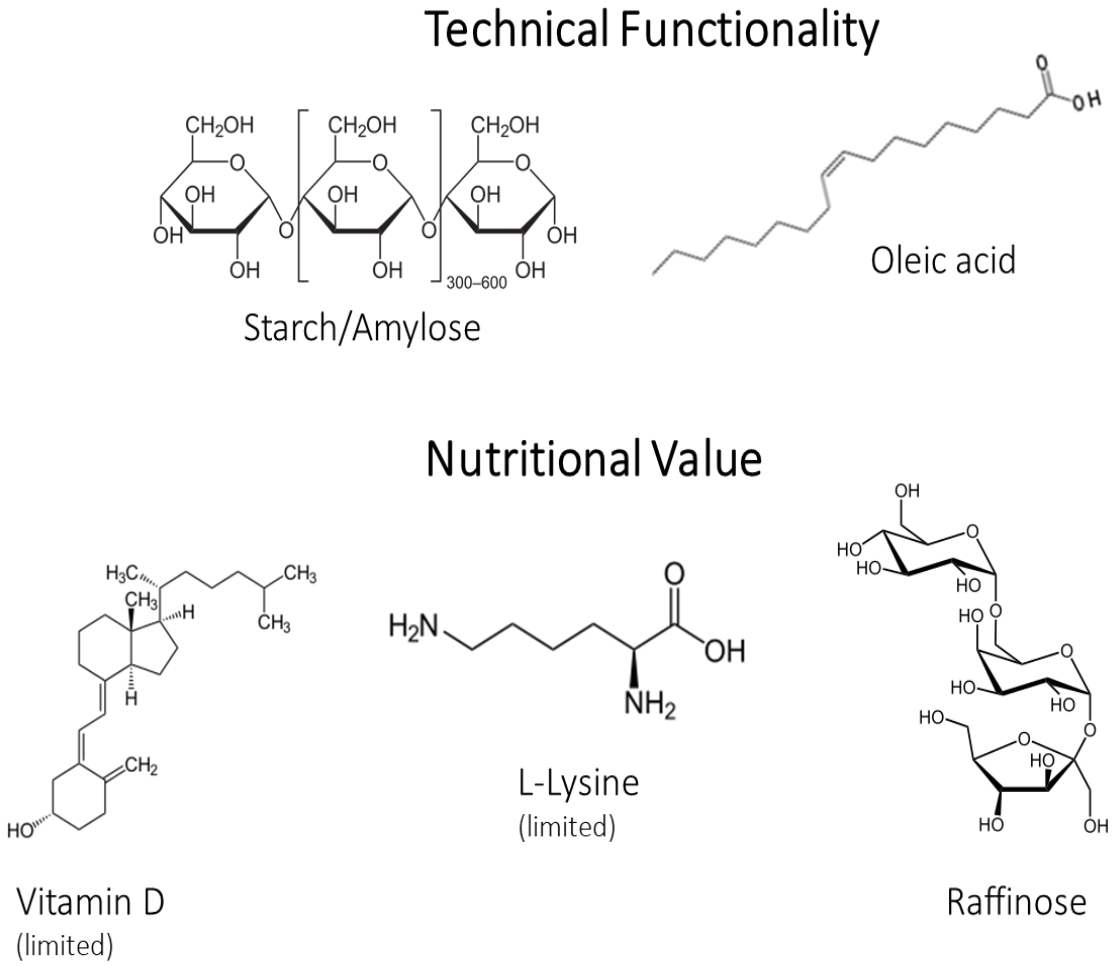

Figure 3. Some typical chemicals that affect the technical properties or nutritional value of plant-based milk alternatives.

1.2.1 Production of Plant-based milk alternative

Plant-based milk alternatives can be produced through various methods, with several shared steps that vary depending on the raw material utilized (Figure 4). Shelled raw materials, such as coconut and walnut, typically require an initial de-shelling process. Some products necessitate dehulling, often achieved through soaking the raw material in water. Roasting is a common practice for peanuts, almonds, hazelnuts, and grains to enhance the emulsion stability index and solubility of protein isolates. Soaking is employed to swell and soften the raw material, facilitating the milling process. Dry milling is seldom used, with wet milling being the more prevalent approach, involving the addition of water to the raw material before grinding. Resuspension in water may be necessary after the milling process. Filtration, utilizing materials such as cheesecloth, muslin cloth, or filtering paper, separates the liquid component from solids. Homogenization is employed to improve the physical stability of the final products. Heat treatment is utilized to extend the shelf life and maintain high product quality.(Tangyu et al. 2019; Aydar et al. 2020.)

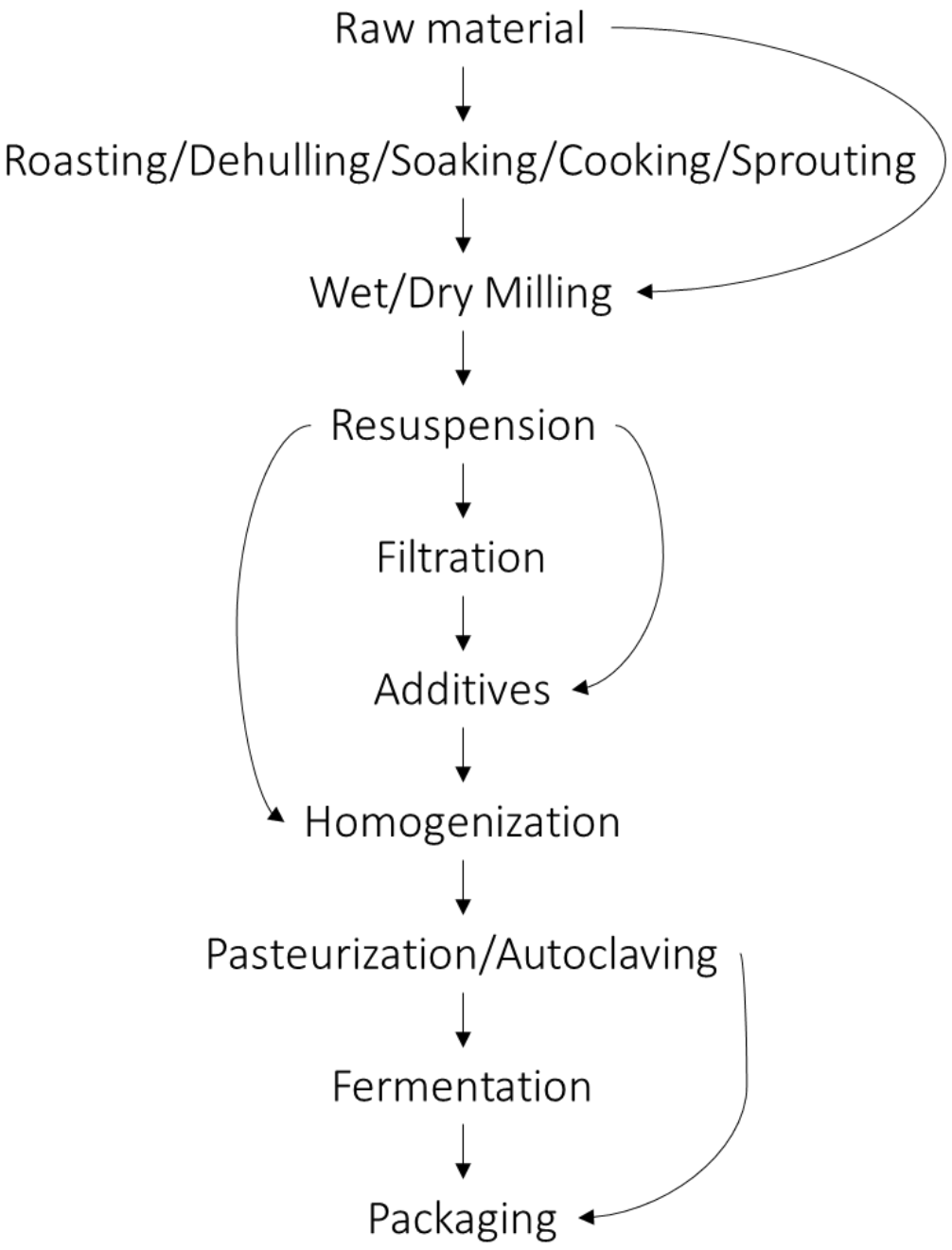

Figure 4. Flow chart for production of plant-based milk alternatives

1.3 Lupin

Lupin, a crop cultivated for food in the Mediterranean region for thousands of years, demonstrates versatility by thriving in a broad geographic range from Iceland to New Zealand(Ishaq et al. 2022). It has the unique ability to grow on marginal agricultural lands in diverse environmental conditions, making it a potential addition to the array of existing food sources. Presently, lupin is cultivated globally, with a significant focus in Australia, primarily for feed but also for food purposes. (Hieta et al. 2010.) The largest number of Lupinus species are

found in the Mediterranean, North and East Africa, and North and South America. (Ishaq et al. 2022.)

Lupins belong to the *Genisteae* family, *Fabaceae*. Around four hundred species of lupin (genus: *Lupinus*) have been found in nature. The most extensively studied and utilized species, including *Lupinus albus L., Lupinus angustifolius L., Lupinus luteus L., and Lupinus mutabilis L*., are commonly referred to as sweet lupins due to their low levels of bitter-tasting alkaloids. This characteristic renders them safe for both animals and humans, eliminating the risk of toxicity. Although lupins have traditionally been used to feed livestock, there is a growing interest in utilizing lupins as a food source due to their unique nutritional value and associated health benefits. (Kohajdová et al. 2011; Khan et al. 2015; Ishaq et al. 2022.)

Historically, *Lupinus albus* (White lupin) was cultivated by classical Egyptians, Greeks, and Romans as feed for animals and flour for bread. *Lupinus luteus* (Yellow lupin) and *Lupinus angustifolius* (Blue lupin) likely have similar extensive cultivation histories. The modern breeding of lupins began in the twentieth century, focusing on three key traits: low alkaloid content, pods retaining seeds for efficient harvesting, and the elimination of seed dormancy. Modern blue and yellow lupin varieties originated in Germany during the First World War, while white lupin crops trace their roots to Australia in the mid-twentieth century. *Lupinus albus* is the most significant lupin seed crop globally, with the majority of production and export centred in Australia*. L. angustifolius*, also known as narrow-leafed lupin or blue lupin, is distributed in Central and Eastern Europe, New Zealand, and Australia, displaying genetic variability suitable for diverse climatic and soil conditions. *L. luteus*, identified by its yellow flowers, exhibits tolerance to acidic and waterlogged soils.(Small 2012; Ishaq et al. 2022.)

While the comprehensive study of bioactive compounds, such as phytochemicals, in edible lupin is ongoing, it is considered cost-effective alternative to other legume crops, particularly soybeans. Lupin shares a similar amino acid profile and protein content with soybeans but surpasses them in dietary fibre. Moreover, lupin can be cultivated across a wider geographical area compared to soybeans, with 80 % of the world's total lupin production occurring in Australia, alongside significant production in Russia and Poland.(Kohajdová et al. 2011; Khan et al. 2015.)

Technological properties of lupin seeds are suitable for industrial processing (Bartkiene et al. 2018). Lupin has been used for example in noodles, pasta, bread, tofu, and tempeh to improve nutritional value. Lupin has been fractioned into dietary fibre to use as a flour and lupin protein isolates have good emulsions and foam-forming properties which can be important aspects of food development. (Khan et al. 2015.)

While lupins thrive as successful protein crops in Australia, their production in Europe falls short of ensuring a stable and ample supply necessary for utilization by the food and feed industry. European lupin production is gradually increasing,

yet it remains insufficient (Figure 5 and Figure 6). In the period between 2010 and 2013, European lupin production only accounted for 17.6 % of the global production during that timeframe. Contrastingly, soya bean stands out as the predominant source of plant proteins for both food and feed. The cultivation of soya bean, along with other beans and peas, surpasses that of lupins in Europe. (Lucas et al. 2015.)

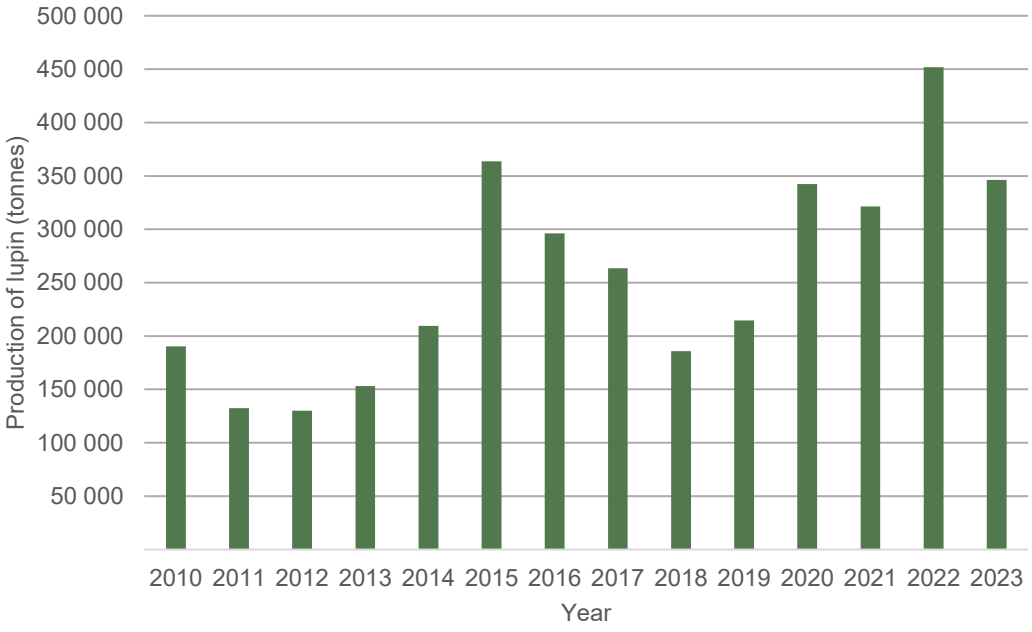

Figure 5. Lupin production in EU in tonnes. Source: European Commission Protein crop statistics.

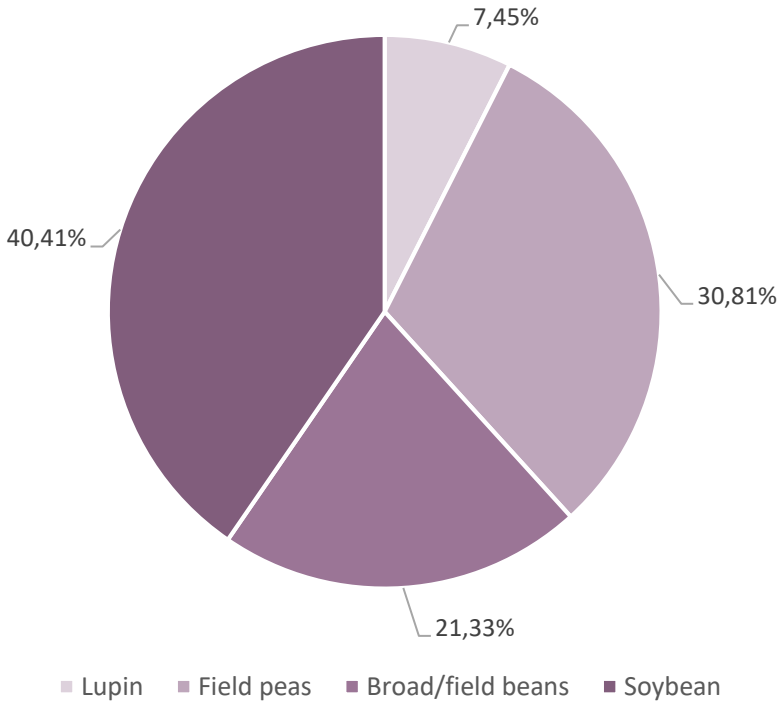

Figure 6. Percentage of protein crop production in EU in 2022. Source: European Commission Protein crop statistics.

1.3.1 Nutritional composition of lupin

The amount of protein in lupin can be approximately two times higher than in more commonly consumed legumes (Figure 7). Protein content varies between lupin species. In addition, characteristics of the growing conditions and soil type effect on the protein content. Lupin seeds contain a lot of good essential amino acids, and they are considered as a good source of lysine (Table 1). Generally, lupines do not have a lot of sulphur-containing amino acids or threonine. (Kohajdová et al. 2011.) The total protein content in lupin seeds is similar to soya bean and significantly higher compared to the total protein content in peas (Bähr et al. 2014).

Table 1: Amino acid composition of *L.angustifolius* seeds *(Sujak et al. 2006)*

Essential amino acids	g/100 g of protein
Lys	4.7
Met + Cys	2.1
Cys	1.4
Thr	3.1
Ile	3.8
Trp	0.7
Val	3.8
Leu	6.6
His	3.1
Phe + Tyr	5.3
Tyr	1.6

Non-essential amino acids	g/100 g of protein
Arg	10.8
Asp	10
Ser	4
Glu	23.1
Pro	3.5
Gly	4.1
Ala	3.1

Dietary fibre and soluble sugar levels of sweet lupin are higher than in most other legumes. In lupin, the sucrose content is the highest among sugars, followed by fructose, raffinose, and glucose. In addition, lupin may contain tetrasaccharide, stachyose, and pentasacharide depending from the variety. (Gdala and Buraczewska 1996; Laaksonen et al. 2021.) Carbohydrate content of lupin seeds is still low compared to carbohydrate content of peas (Bähr et al. 2014). Lupin also contains minerals (Table 2) and phytochemicals such as bioactive peptides, alkaloids, polyphenols, phytosterols, and tocopherols, although detailed information is lacking, especially in terms of the effect of technological processing on the phytochemical composition. Studies have shown that lupin has lower levels of undesirable constituents like phytic acid and oligosaccharides than other legumes. (Kohajdová et al. 2011; Khan et al. 2015.)

Liquid samples that are produced from lupin seeds contain citric and malic acids. In addition, there may be low contents of maleic, succinic, lactic, and fumaric acids. Sucrose is the main sugar in the liquid lupin seed sample. Glucose and raffinose also have a significant content. Other sugars that studies have found in lupin seed samples are galactose, fructose, maltose, and mannitol.(Laaksonen et al. 2021.)

The fiber and protein contents of lupin contribute to health benefits, particularly in managing obesity and type 2 diabetes. Lupin seeds contribute to reduced risk of cardiovascular diseases and metabolic syndrome. Additionally, the phytochemicals in lupin exhibit antioxidant, antihyperlipidemic, and anti-inflammatory properties, acting against chronic diseases. However, it is essential to note that the phytoestrogens in lupin may have antiestrogenic properties, potentially leading to adverse health effects. (Khan et al. 2015; Bartkiene et al. 2018.)

Lupin seed flour has demonstrated health benefits, including preventing hypercholesterolemia and hypertension. Human studies suggest that either the fibre or protein in lupin seeds may play a role in preventing hypercholesterolemia. Hypoglycaemic activity is likely attributed to specific proteins absorbed intact from the intestine, while hypocholesterolaemia and hypotensive activities may be related to specific peptides released during digestion. However, these findings are yet to be fully confirmed in studies (Arnoldi et al. 2015.)

Lupin polyphenols also have their own health benefits. Polyphenols are strong antioxidants that can prevent lipid oxidation and atherosclerosis formation and are also efficient ACE-inhibitors. (Arnoldi et al. 2015.)

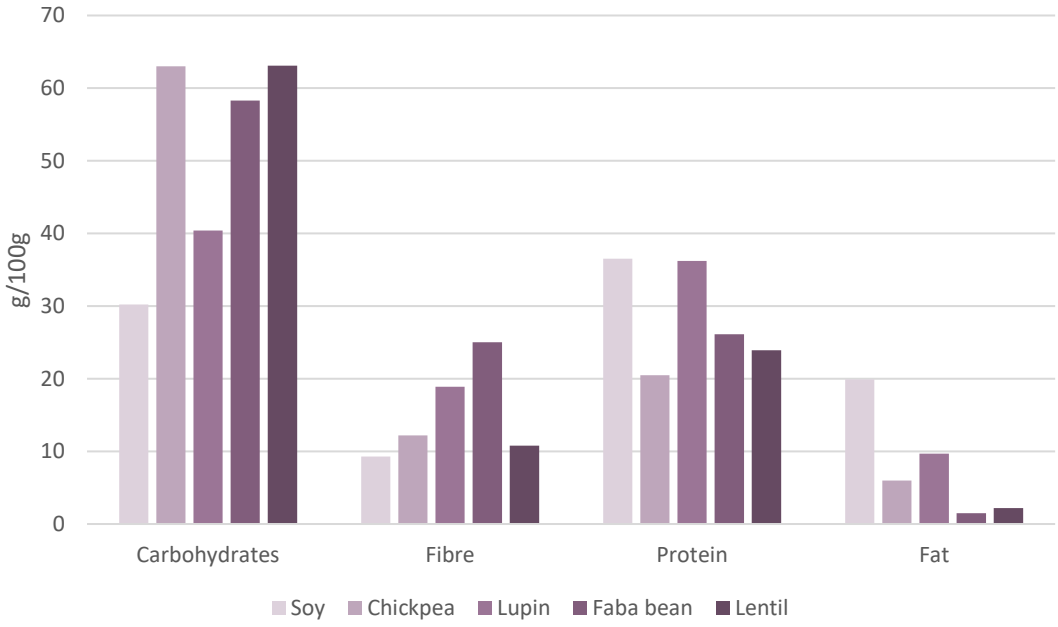

Figure 7. Carbohydrate, fibre, total protein, and total fat content of some common legumes *(Cichońska and Ziarno 2021)*.

Table 2. Nutritional facts of lupin flour (Makealupiinijauho, Koivunalhon Luomutila, Lieto, Finland) per 100 g.

Total Fat	8.1 g
Saturated fat	1.6 g
Monounsaturated fat	2.4 g
Polyunsaturated fat	3.7 g
Omega-3	0.4 g
Omega-6	3.3 g
Carbohydrates	41.4 g
Dietary fibre	30.8 g
Sugars	3.03 g
Protein	38.3 g
Salt	0.02 g
Phosphor	470 mg
Potassium	1200 mg
Calcium	180 mg
Magnesium	250 mg
Manganese	1.1 mg
Iron	3.4 mg

1.4 Fermentation

Fermentation is one of the oldest food processing technologies in the world. It is a natural bioprocess that is usually used to preserve food. Fermentation increases shelf life and microbiological safety of food. It can also be used to make some food more easily digestible and reduce the toxicity and improve the nutritional values and sensory properties. (Caplice and Fitzgerald 1999; Nowacka et al. 2021.)

During fermentation carbohydrates oxidate and related to derivatives end-products are generated. End-products are usually acids, alcohol, and carbon dioxide. In most fermented foods, lactic acid bacteria are used in fermentation process, but also other types of microorganism can be used. Fermentation leads to different effects on the functional, nutritional or sensory properties of the final product depending on the used microorganism. The end-products in lactic acid bacteria fermentation contributes to preservation, flavour, aroma, and texture. Fermentation increases the nutritional value of food by increasing digestibility. (Caplice and Fitzgerald 1999; Leonard et al. 2021.)

Fermentation may cause various changes in the legume composition. Fermentation can alter legumes amino acid composition by amino acid synthesis and affect levels of trypsin and chymotrypsin inhibitors. Fermentation may also lower α-galactoside levels. Lower levels of α-galactosides may reduce the induction of abdominal discomfort after pulse ingestion. Studies have shown that depending on pulse species and lactic acid bacteria used in fermentation, α-

galactoside may reduce up to 64 %. Fermentation can also eliminate raffinose and stachyose almost completely. Phenolic compounds have various health effects which makes them desirable in pulses. Fermentation can positively affect polyphenol composition of pulses. Phenolic compounds have antimicrobial, antioxidant, and phytoestrogenic properties. Studies have shown that fermentation can increase polyphenol content and, thus, increase antioxidant capacity. (Boeck et al. 2021.)

1.4.1 Lactic acid bacteria fermentation

Lactic acid bacteria (LAB) are group of Gram-positive, non-spore forming, anaerobic but oxygen tolerant bacteria which produce lactic acid as the major metabolic end product when fermenting with carbohydrates. They can be found from nature in plants, milk, fermented foods, and mucosal surfaces of the human body. The major genera that comprise of LAB are *Lactococcus, Lactobacillus, Leuconostoc, Pediocoocus,* and *Streptococcus.* LAB are usually mesophilic but can grow at temperatures from 5 °C to 45 °C. Optimal pH for growing depends on strain. Majority of LAB strains grow at pH 4.0−4.5 but some are active at pH 9.6 and some are pH 3.2. Generally, LAB strains are weakly proteolytic and lipolytic and usually require preformed amino acids, purine, and pyrimidine vases and vitamin B for growth. In studies, lupin samples' pH values have been around 5.0−5.9 at the start of fermentation and the values have decreased during fermentation. (Caplice and Fitzgerald 1999; Peng et al. 2020.)

Lactobacillus perform homolactic fermentation. In pure lactic acid fermentation, LAB use glucose as a carbon source to produce pyruvate through glycolysis and then in lactase dehydrogenase lactic acid is produced. In theory, 1 mole of glucose produces 2 moles of lactic acid (Figure 8). (Nelson et al. 2017)

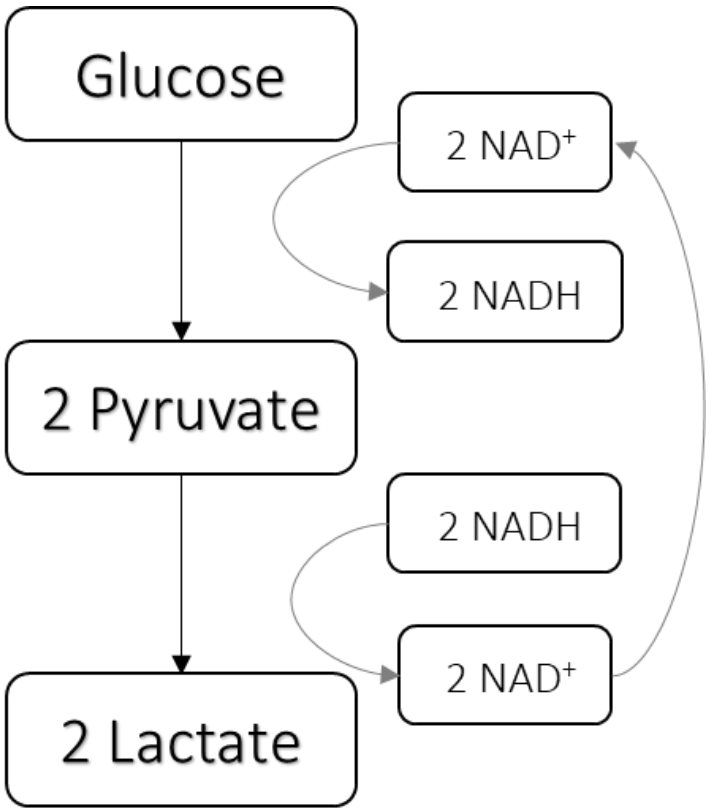

Figure 8. Flow chart of lactic acid fermentation

LAB have the ability to start a fast acidification of dairy products. They also produce other molecules, such as acetic acid, aroma compounds, and enzymes, and also a variety of antimicrobial substances, such as organic acids, diacetyl, hydrogen peroxide, and bacteriocins. LAB are widely used in food fermentation. Most of the produced antimicrobial substances has potential to work as food preservatives or as therapeutic or bio-controlling agents. (Peng et al. 2020; Barbosa et al. 2021; Nowacka et al. 2021.)

LAB fermentation has been used for ages as a food preservation method and it has an essential place in food industry. LAB fermentation has an ability to control the fungal growth and remove the food-contaminating substances like mycotoxins. LAB´s antimicrobial activity has been explained by the acidic environment from production of organic acids, the competition for nutrients and the formation of antimicrobial compounds.(Peng et al. 2020.) In general, LAB fermentation is used for dairy products but it has shown potential for using it in plant-based products. LAB fermentation improves digestibility of plant proteins and reduces the anti-nutrient content. (Laaksonen et al. 2021.)

Fermentation of plant-based products changes the consistency to resemble dairy products. Fermentation also helps to lower the antinutritional components of plant-based ingredients. (Alcorta et al. 2021.) LAB fermentation of lupin seeds has shown to affect sugar, acid, and volatile compound contents which affects the sensory quality. Fermentation decreases aldehydes which decreases odours

that are not wanted for food products. Fermentation increases contents of lactic acid and volatile acids on the dairy analogue produced from lupin. However, effects of fermentation depends on the used LAB strain. (Laaksonen et al. 2021.)

Studies have shown that commercial starter LAB cultures possess many metabolic properties including acidification activity, proteolytic activity, and antagonistic activity. Its properties affect to the nutritional values and organoleptic attributes of the final products. (Peng et al. 2020.)

LAB can also be used as probiotics because they are resistant to the gastrointestinal acidity, able to adhere to the intestinal mucosa, improve the intestinal microbiota, and reduce the growth of undesirable bacteria. Moreover, some LAB strains have shown the ability to reduce cholesterol. (Peng et al. 2020.)

LAB fermentation produces lactic acid as a final product. Lactate is the conjugate base of lactic acid. Lactate may regulate critical functions of several parts of immune system, such as macrophages and dendritic cells. Lactate can influence cellular activities by three independent ways at least. It can modulate gene expression through modification of histone deacetylase activity, trigger different signalling pathways by GPR81, or induce changes in metabolic pathways. With these cellular processes, different functional effects are achieved, which means that lactate may contribute to the properties of fermented foods. (Garrote et al. 2015.)

For food fermentations, LAB should have some important metabolism characteristics. It should have ability to produce acid and aroma, ability to hydrolyse protein, ability to produce viscous exopolysaccharides and ability to inhibit bacteria. LAB can hydrolyse polysaccharides with α-glycosidic bonds and improve the in vitro digestibility of protein by increasing bioactive diversity by editing the proteolytic system. LAB has been shown to synthesis many different substances in food. Lactic acid is the most typical substance that LAB fermentation synthetises but also other organic acids have been shown to be able to produce such as formic acid, acetic acid, propionic acid, butyric acid, and succinic acid. (Wang et al. 2021.)

Polysaccharides in plants include for example starch, cellulose, and hemicellulose. In fermented food, the decomposition of polysaccharides can provide energy for LAB and also provide a variety of beneficial substances for human beings. Different LAB can metabolize different polysaccharides, which determines the different applications in food industry. The degradation of polysaccharides in LAB fermentation can produce monosaccharides or lactic acid. Some genera of LAB are regarded as probiotics in intestine. *Lpb. plantarum* has shown to produce amylase to hydrolyse starch into dextrin and finally into glucose.(Wang et al. 2021.)

1.4.2 Lactiplantibacillus plantarum

Lactiplantibacillus plantarum (Lpb. plantarum) is part of the genus *Lactobacillus*. *Lpb. plantarum* species have been used as starter culture in the fermentation process of many fermented foods such as sauerkraut, table olives, and dairy products. Because of the long history of safe uses, *Lpb. plantarum* is included in the qualified presumption of safety recommendation of European Food Safety Authority. Fermentation process improves both food quality and safety and also prolong the shelf life of final products by inhibiting food spoilage microbes.(Barbosa et al. 2021; Garcia-Gonzalez et al. 2021.)

Ingestion of *Lactiplantibacillus* has been proposed to have various health benefits such as modulating immune system and improving resistance to infectious diseases (Barbosa et al. 2021). *Lactobacillus* is considered to be a probiotic and that way *Lb. plantarum* may have probiotic potential. However, *Lpb. plantarum* must have a high gastrointestinal survival rate to meet requirements for probiotic. (Garcia-Gonzalez et al. 2021.)

Probiotic bacteria exert their health benefits through various common mechanisms, including the production of bioactive molecules, modulation of the immune system, exclusion or inhibition of pathogens, and enhancement of the intestinal epithelial barrier through increased mucin production. Additionally, probiotic bacteria contribute to the modulation of commensal microbiota. One notable probiotic species, *Lpb. plantarum* (*Lpb. plantarum*), exhibits broad-spectrum antibacterial activity against numerous food spoilage microbes. Consequently, *Lpb. plantarum* strains are deemed suitable for application in the food industry as bio-preservatives. Studies have demonstrated the inhibitory effects of *Lpb. plantarum* against both Gram-positive and Gram-negative bacteria. Furthermore, *Lpb. plantarum* exhibits effective antifungal activity against various yeast and mold species. This antimicrobial activity primarily stems from the production of compounds such as organic acids and hydrogen peroxide. (Garcia-Gonzalez et al. 2021.)

1.4.3 Lupin fermentation with lactic acid bacteria

Table 3 shows previous studies about LAB fermentations of lupin. Lupin fermentation have been tested with many different LAB strains and different effects have been analysed. Most studies have focused on the effects of fermentation on the protein content or the amino acid composition or the sensory properties of fermented lupin products. In addition, some previous studies have researched the impact of LAB fermentation on sugar and acid concentrations.

Table 3. An overview of previous studies of lactic acid bacteria fermentation of lupin

Study	*Ingredients*	*Effect*
The influence of lactic acid fermentation on functional properties of narrow-leaved lupine protein as functional additive for higher value wheat bread (Klupsaite et al. 2017)	Lupin (*L. angustifolius)* seeds *P. pentosaceus*	Total protein content decreased by 19% after 72 h of fermentation. pH value decreased from 5.21 to 3.11 after 72 h of fermentation.
Impact of lactic acid fermentation on sensory and chemical quality of dairy analogues prepared from lupine (Lupinus angustifolius L.) seeds (Laaksonen et al. 2021)	Sweet lupine seeds (*Lupinus angustifolius L.)* Five different LAB starters or starter mixtures	Increased lactic acid content and reduced sucrose content. pH decreased from 5.9 to below 5 during 48 h of fermentation. Sourness and ´vinegar´ odour increased. ´beany´ odour and flavour and unpleasantness of flavour decreased.
Solid state fermentation with lactic acid bacteria to improve the nutritional quality of lupin and soya bean (Bartkiene et al. 2015)	Lupin seeds and soya beans. *Lactobacillus sakei, Pediococcus acidilactic* and *Pediococcus pentosaceus.*	Protein digestibility was found higher on average by 18.3 %. Produced mainly L-lactic acid. pH decreased on average by 4.3 % after 24 h of fermentation.

Tecnctional and Sensory Properties ofFermented Lupin Protein Isolates (Schlegel et al. 2019)	Lupin protein isolate made from *Lupinus angunstifolius.* Nine different LAB strains.	Fermentation maintains functional properties and improves sensory properties of lupin protein isolates. pH values decreased from 6.6 – 6.0 to 5.2−3.9 after 24 h of fermentation.
Effect of Gemination and Fermentation on Carbohydrate Composition of Australian Sweet Lupin and Soybean Seeds and Flours (Kaczmarska et al. 2017)	Australian sweet lupin seeds and lupin flour YO-MIX yogurt culture (*Lactobacillus delbrueckii subo. bulgaricus* and *Streptococcus thermophilus*) and spontaneous fermentation	Sucrose content decreased when fermented with yogurt culture but increased during spontaneous fermentation. Both glucose and fructose increased during fermentations.

1.5 Bioreactor

Bioreactor is a device or vessel where biological reaction takes place. One of the most common bioreactor type is stirred tank reactor and it is used almost universally for fermentations. Stirred tank reactors have a agitator or impeller which performs heat transfer, aeration, and mixing for homogenization. It has many advantages such as existing industrial capacity, proven performance, and ease of scale-up and control. Thus, it is one of the most used bioreactor type.(Lidén 2002; Wang and Zhong 2007.)

Bioreactor provides optimal environment for the reaction by maintaining control over factors such as temperature, pH, agitation and nutrient supply. This ensures consistent and reproducible conditions. Bioreactors are used in a wide range of applications for example in pharmaceutical processes, wastewater treatment, and food fermentations. In food industry, bioreactors can be used for example for fermenting food products or producing enzymes or probiotics. (Lidén 2002; Wang and Zhong 2007.)

1.5 Aim of the study

The aim of this study was to successfully ferment dairy alternative made from lupin with LAB using a bioreactor. The focus was on understanding the changes on simple sugars and organic acids contents. Utilizing the bioreactor provides a controlled and consistent environment, ensuring more reliable information about the effects of fermentation.

2 Materials and Methods

2.1 Preparation of the test liquid lupin fraction

One liquid lupin fraction sample for test fermentations was prepared from lupin (*Lupinus angustifolius L.*) seeds using a later described method by Laaksonen et al. (2021), but on a smaller scale and without barley starch. Seeds were rinsed and soaked overnight in tap water. Soaked seeds were drained, dehulled, and rinsed. Dehulled seed were grounded with tap water in 1:1.12 w/w to form a slurry which was then filtered through mesh cheese cloth. The filtered liquid fraction was centrifugated at 5600 x g for 10 minutes (Avanti JXN-26, Beckman Coulter, USA) and liquid fraction was collected and pasteurised at 95 °C for 30 seconds. Liquid lupin fraction was stored in freezer until fermentation.

Commercially bought sweet lupin flour (Makealupiinijauho, Koivunalhon Luomutila, Lieto, Finland) was mixed with tap water in a 1:10, 1:15, and 1:20 ratio using a blender for 30 seconds. Blended product was filtered through cheese cloth and centrifuged at 5000 x g for 5 minutes. Liquid fraction was collected and pasteurised at 95 °C for 30 seconds. Liquid lupin fraction was stored in freezer until fermentation.

The differences in sample preparation methods are shown in Figure 9.

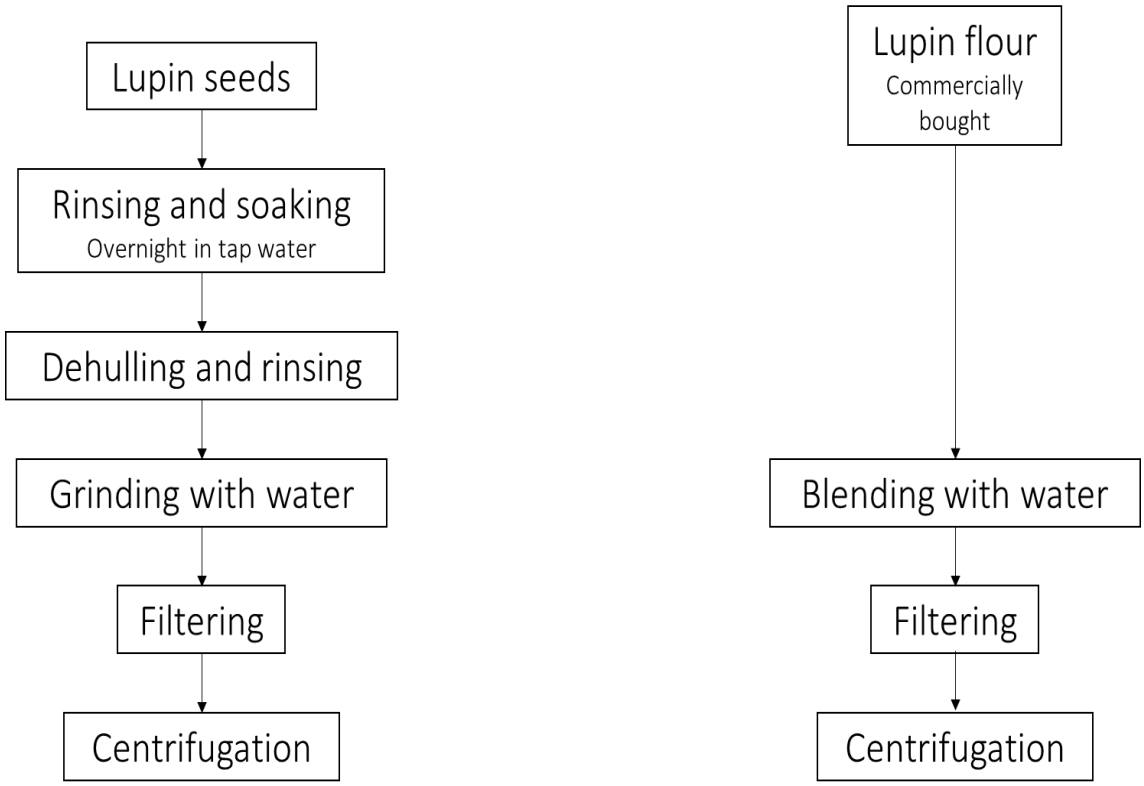

Figure 9: Flow chart of liquid lupin fraction preparation methods

2.1.1 Preparation of the final liquid lupin fraction

Sweet lupin flour was mixed with tap water in a 1:10 ratio using a blender. Blended product was centrifuged at 5000 x g for 10 minutes. Liquid fraction was collected and stored in freezer until fermentation.

2.2 Fermentation of lupin

2.2.1 Lactic acid bacteria

Bacteria strains from MRS glycerol stock were preincubated 24 h in MRS broth (acumedia Neogen, USA) at 30 °C. Bacteria cells were washed twice by centrifuging (3500 rpm, 20 min, 8 °C) and suspending pellet to 0.9 % saline. Washed bacteria pellet was suspended in 0.9% saline and used for fermentations. Dilution series were made from bacteria to determine CFU/mL. Dilutions were spreaded to MRS plates and incubated at 30 °C for 48 h. Colony count was counted from a plate with 30−300 colonies.

2.2.1 Test fermentations

Fermentation was first verified in small batches (10 mL) for all of the samples by measuring pH after 24 h, 48 h, and 72 h of incubation at 30 °C. *Lactiplantibacillus plantarum* 100813*, Lactiplantibacillus plantarum* 10492, and *Lactiplantibacillus plantarum* 20174 were the LAB strains used in test fermentations. Bacteria concentration before fermentation was approx. 1 x 10^8 CFU/mL. Samples from 0 h, 24 h, 48 h, and 72 h were collected and frozen -20 °C until further analysis.

2.2.2 Bioreactor

The final fermentations were conducted using a bioreactor (Minifors 2, Infors HT, Switzerland). Vessel capacity was 1.5 L. The bioreactor was autoclaved with liquid lupin fraction prior fermentation. Throughout the fermentation process the temperature was monitored continuously and controlled. Stirring was maintained consistently during fermentation. pH measurements were recorder throughout the fermentation. Samples were extracted during fermentation using aseptic sampling system (Super Safe Sampler, Infors HT, Switzerland). The bioreactor system is simplified illustrated in Figure 10.

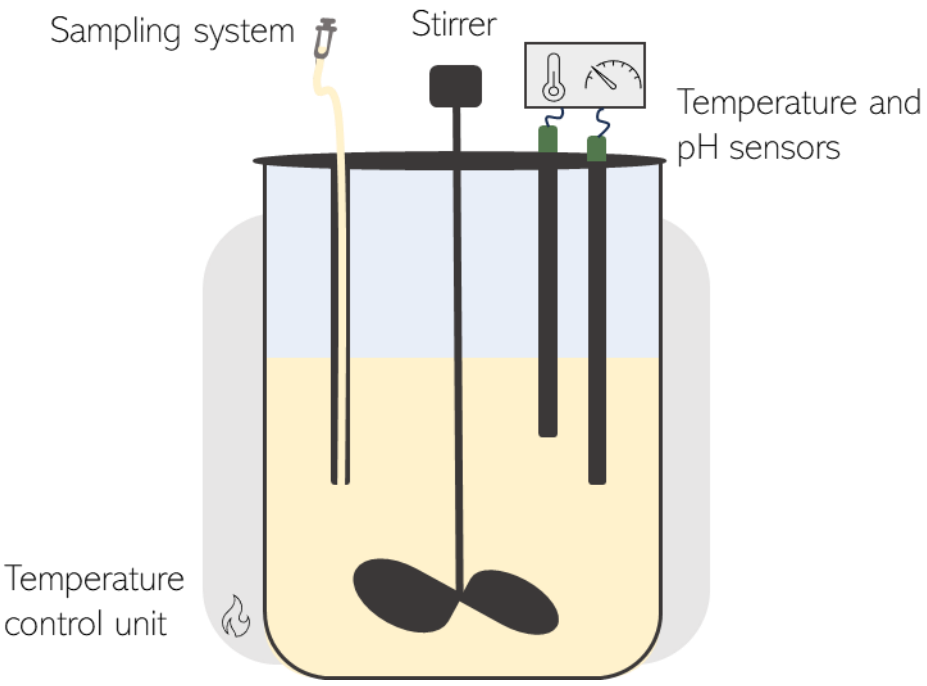

Figure 10: Structure of bioreactor system

2.2.3 Final fermentations

Final fermentations were done for 1:10 liquid lupin fraction using *Lactiplantibacillus plantarum* 10492 and *Lactiplantibacillus plantarum* 20174 as LAB strains. Bacteria strains were treated in the same way as before. Liquid lupin fraction was added to bioreactor and whole system was autoclaved. After autoclave, bacteria were added to bioreactor. Bacteria concentration before fermentation was approx. 1 x 10^8 CFU/mL. Bioreactor was kept at 28°C and stirring was 100 rmp/min. Samples for sugar and acid analysis were collected at 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h. Samples were frozen -20 °C until analysing.

2.3. Analysis of sugars and acids

Sugar and organic acid composition of fermented lupin dairy analogues was determined using a gas chromatography-flame ionization detector (GC-FID). GC-FID samples were prepared from the test fermentation samples by adding 0.25 mL of xylitol and tartaric acid as standards to 0.25 ml of fermentation sample. Then it was diluted to final volume of 5 mL with ultrapure water. Samples were filtered with syringe filters (0.45 µm RC) and 300 µL of filtration was pipetted to autosampler vial.

The final sample preparation was conducted by adding 2 g of sample to 10 mL flask. Internal standards, xylitol and tartaric acid (0,5 mg of each) were added, and flask was filled with purified water. The sample was centrifuged at 4000 g for

5 min and supernatant was collected. Supernatant was filtered by 0.2 µm wwPTFE filter. 300 µL was transferred to autosampler vial.

Samples were evaporated to dryness under nitrogen flow at 50 °C for 45 min and kept in desiccator over P2O5 until analysing. TriSil reagent (500 µL, Thermo-Fisher, Waltham, MA) was added and mixed for 5 min in shaker. After mixing samples were incubated at 60 °C for 30 min.

GC-FID analysis were carried out using a Shimadzu GC-2010 Plus with AOC-20i autosampler and FID (Shimadzu Europe, Duisburg, Germany). Column was SPB^{TM}-1 (30 m x 0.25 mm ID, liquid film 0.25 µm, Supelco, Bellefonte, PA, USA). The injector was 210 °C and the detector 300 °C. The oven temperature program consisted of the following steps: holding an initial temperature of 100 °C for three minutes, increasing it to 205 °C at a rate of 4 °C/min, holding it at 255 °C at a rate of 40 °C/min for four minutes, and holding it at 300 °C at a rate of 4 °C/min for 30 minutes. 2.05 mL/min of helium was utilized as the carrier gas, moving at a constant linear speed of 44.08 cm/sec. Internal standards xylitol and tartaric acid was used for quantitative calculations.

General process of the whole experiment is in Figure 11.

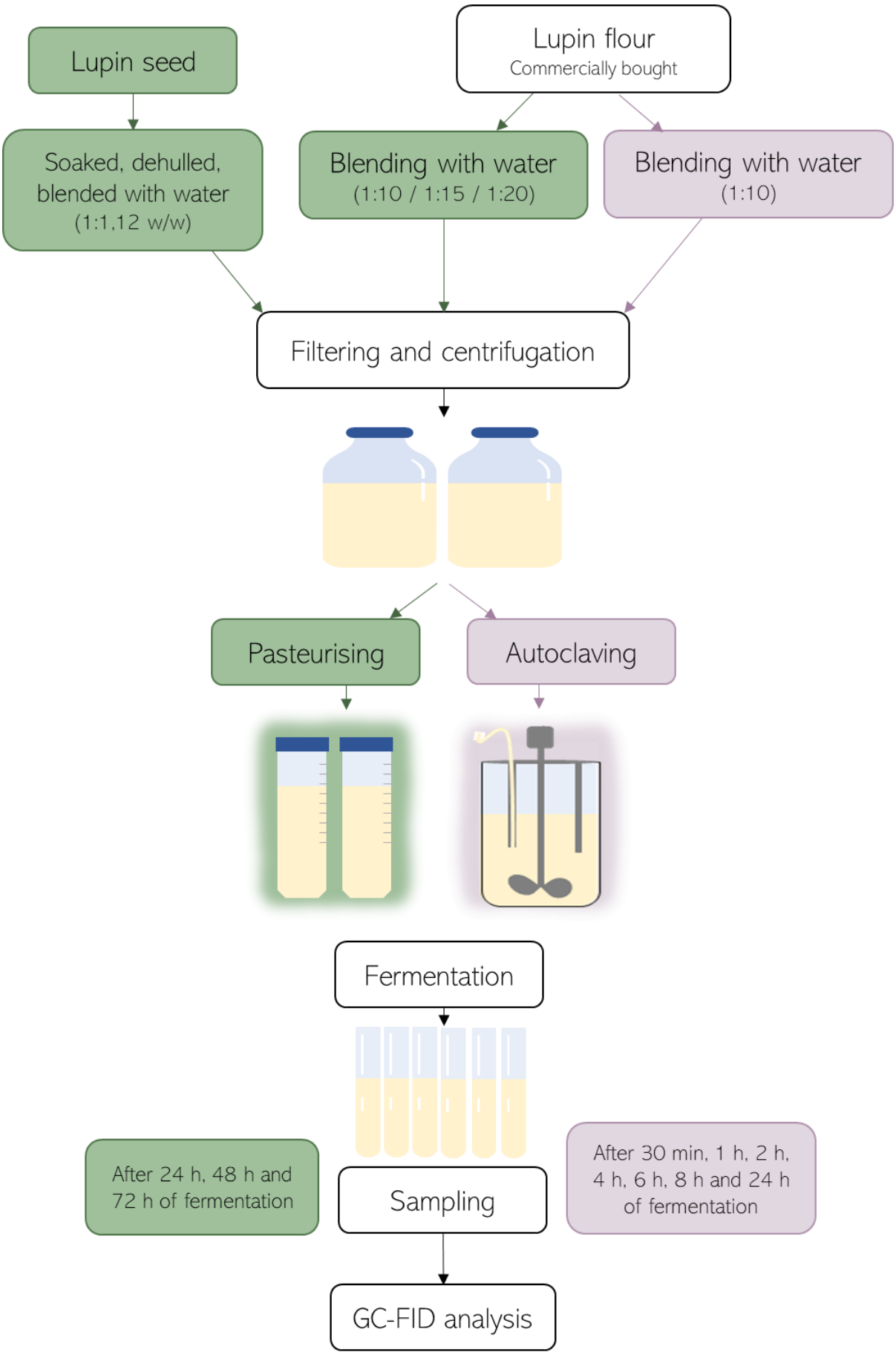

Figure 11: Experimental design flowchart. Test fermentations are represented in green and final fermentations are in purple.

3 Results and discussion

3.1 Test fermentation

The test fermentations were done to see which dairy analogue made from lupin flour and water was closest to dairy analogue made from lupin seeds with method from Laaksonen et al. (2021). GC-FID analysis of sugars and organic acids were done for all samples without fermenting and fermenting for 24 h, 48 h and 72 h. Test fermentations also revealed how fast the fermentation happens in liquid lupin fraction.

1:10 lupin flour-to-water ratio was the most similar to the liquid lupin fraction made form seeds, so we decided to continue our study with that sample preparation method (Figure 12). Using the seeds and the sample preparation method of Laaksonen et al. (2021) was found to be too difficult to execute for small sample batch and with available equipment.

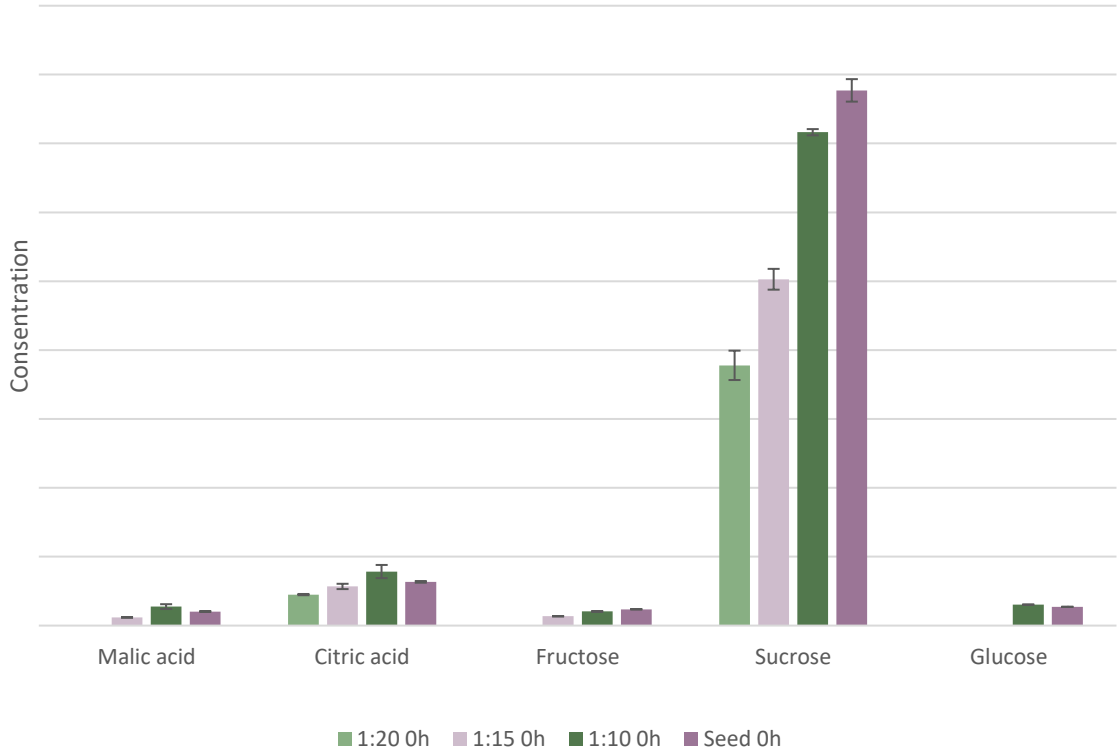

Figure 12: Liquid lupin fraction ratio tests before fermentation

The GC sample preparation process used for the test fermentations was not precise enough. GC-FID could only detect fructose, sucrose, glucose, and lactic acid from the samples. The first GC sample preparation method was tested because we had too small sample quantity to use GC sample preparation protocol from Laaksonen et al. (2021). The protocol was modified to fit smaller

sample quantity. One of the liquid lupin fractions was tested with that method to see if we could implement the method in smaller scale. With the modified protocol GC-FID could detect malic acid, citric acid, quinic acid, and fructose, glucose and sucrose more reliably. Figure 13 shows a chromatogram of lupin dairy analogue before fermentation and after 24 h of fermentation. Malic acids peak looks similar before and after fermentation. Lactic acid peak has grown, and sucrose peak is reduced. Glucose can be found before fermentation but not after fermentation.

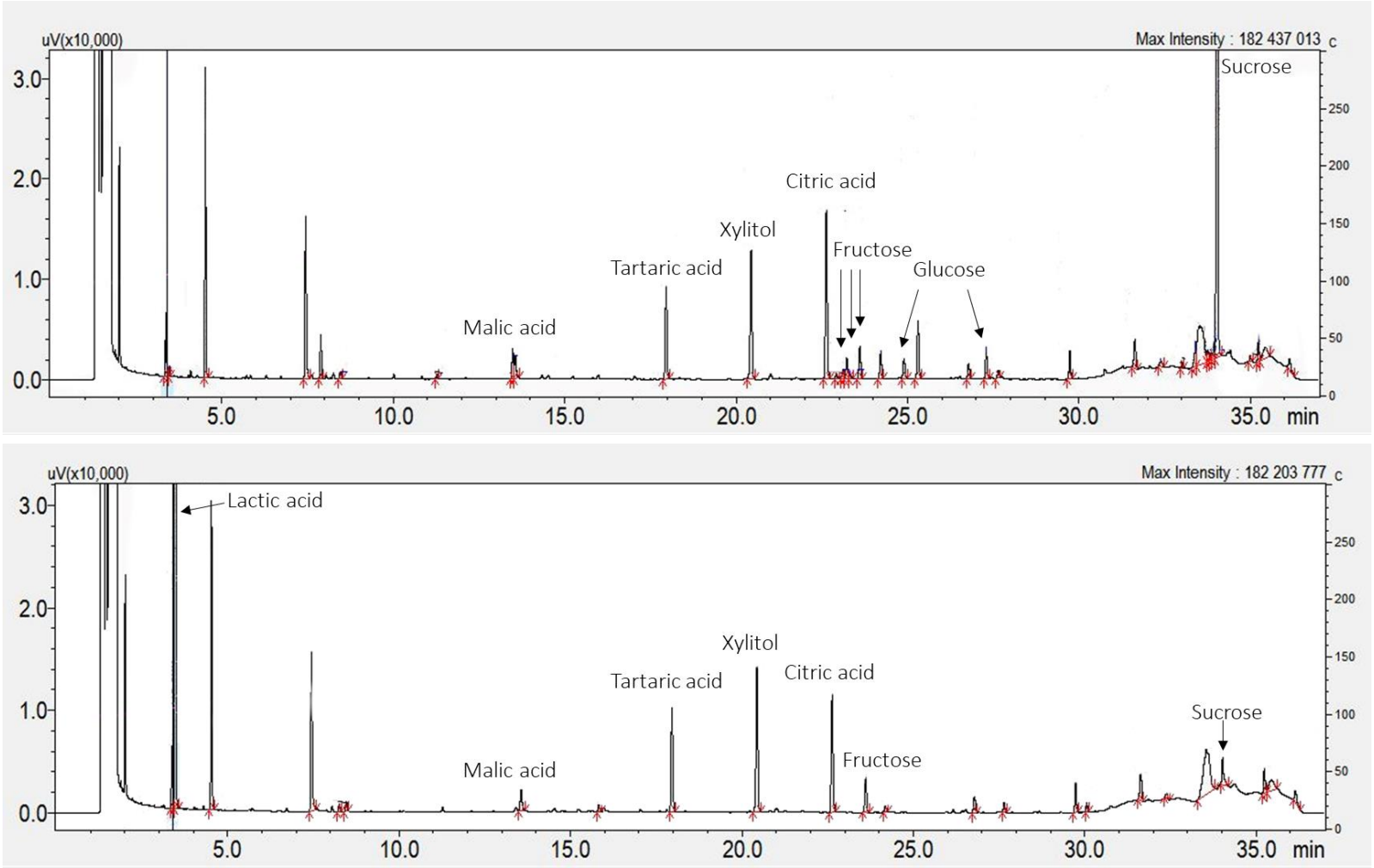

Figure 13: GC-FI chromatogram of lupin dairy analogue before fermentation and after 24 h fermentation

3.2 Fermentations with bioreactor

In the chromatogram of the sample taken before fermentation, sucrose, glucose, fructose, citric acid, and malic acid were identified from their distinct peaks. In the chromatogram of the after fermentation sample, sucrose, fructose, citric acid, malic acid, and lactic acid were identified. Before fermentation, sucrose caused the highest peak, whereas after fermentation, lactic acid showed the highest peak. In the post-fermentation chromatogram, there is only one peak for fructose and no observable peak corresponding to glucose, whereas before fermentation, there were three distinctive peaks of fructose and two peaks of glucose.

Table 3 shows mean of sugar and acid concentrations before and after fermentations. Before fermentation, sucrose comprised the majority of the detected sugars, accounting for over 90% of the total. Fructose constituted approximately 3%, while glucose made up 5% of the sugar composition. Following 24 hours of fermentation, the concentration of fructose remained stable, indicating a lack of significant changes in its content. Glucose was not detectable post-fermentation, and the sucrose concentration had declined to a level equivalent to that of fructose. Both glucose and sucrose showed significant difference between unfermented and fermented samples.

Table 4 Sugar and acid content after 24 h of fermentation.

g/10mL	Unfermented	*Lbp. Plantarum* 10492	*Lbp. Plantarum* 20174
Lactic acid	0.5 ± 0.0 ^{B}	130.6 ± 5.7 ^{A}	124.8 ± 6.3 ^{A}
Malic acid	2.0 ± 1.0	1.2 ± 0.2	1.3 ± 0.0
Citric acid	16.3 ± 1.8 ^{A}	12.0 ± 1.2 ^{A}	9.0 ± 0.8 ^{B}
Quinic acid	1.4 ± 0.3	0	0
Total acids	20.3 ± 1.8 ^{B}	144.3 ± 4.9 ^{A}	135.3 ± 7.1 ^{A}
Sucrose	84.9 ± 8.2 ^{A}	3.8 ± 2.3 ^{B}	2.1 ± 0.9 ^{B}
Fructose	2.3 ± 1.1	2.4 ± 0.1	2.3 ± 0.0
Glucose	4.3 ± 0.1	0	0
Total sugars	91.5 ± 7.4 ^{A}	6.2 ± 2.3 ^{B}	4.4 ± 1.0 ^{B}

Significant differences between samples, if detected, are shown with letters (T-test, $p < 0.05$)

In the pre-fermentation samples, there was a slight presence of quinic acid, but post-fermentation, it was no longer detectable. Additionally, the concentrations of malic acid and citric acid experienced a minor decrease during fermentation (Figure 14). However, the decrease of malic acid was not significantly different between unfermented and fermented samples. Citric acid did not have significant

decrease when fermented with *Lbp. Plantarum* 10492. Fermentation with *Lbp. Plantarum* 20174 had significant decrease of citric acid. A minimal amount of lactic acid was detected in the samples before fermentation, as anticipated (Figure 15). Following fermentation, the concentration of lactic acid increased significantly, reaching over 1200 mg/100mL with both used LAB strains. The results were consistent with those reported in the literature.(Laaksonen et al. 2021) When comparing the fermentation outcomes between different bacterial strains, the only statistically significant difference is observed in citric acid. Other sugar and acid concentrations are statistically similar among the fermentation end products of the bacterial strains.

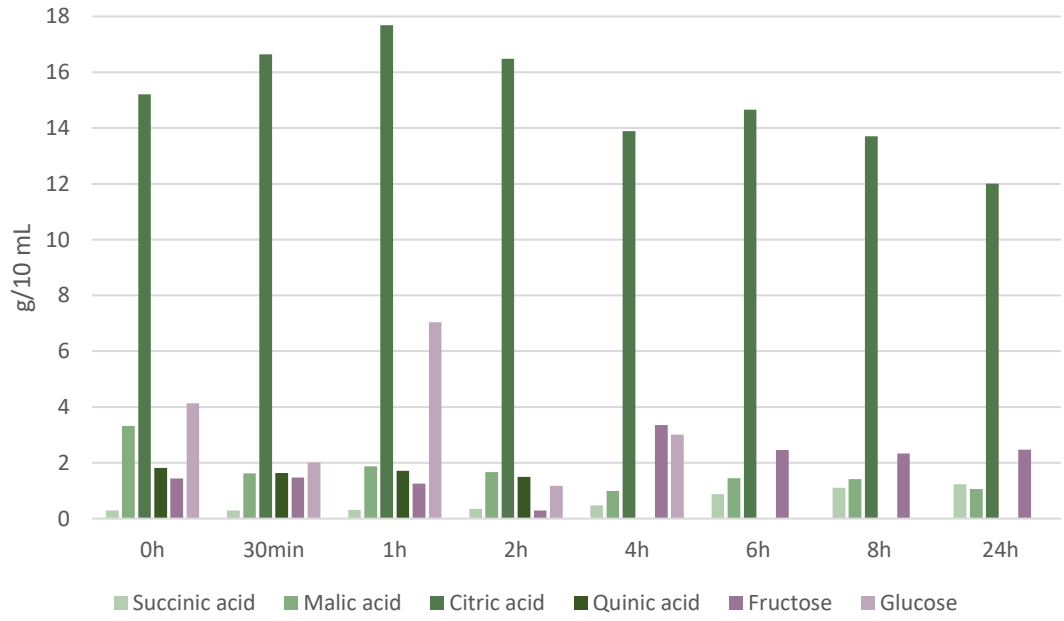

Figure 14 Mean of succinic acid, malic acid, citric acid, quinic acid, fructose and glucose contents during fermentation with *Lbp. Plantarum* 10492.

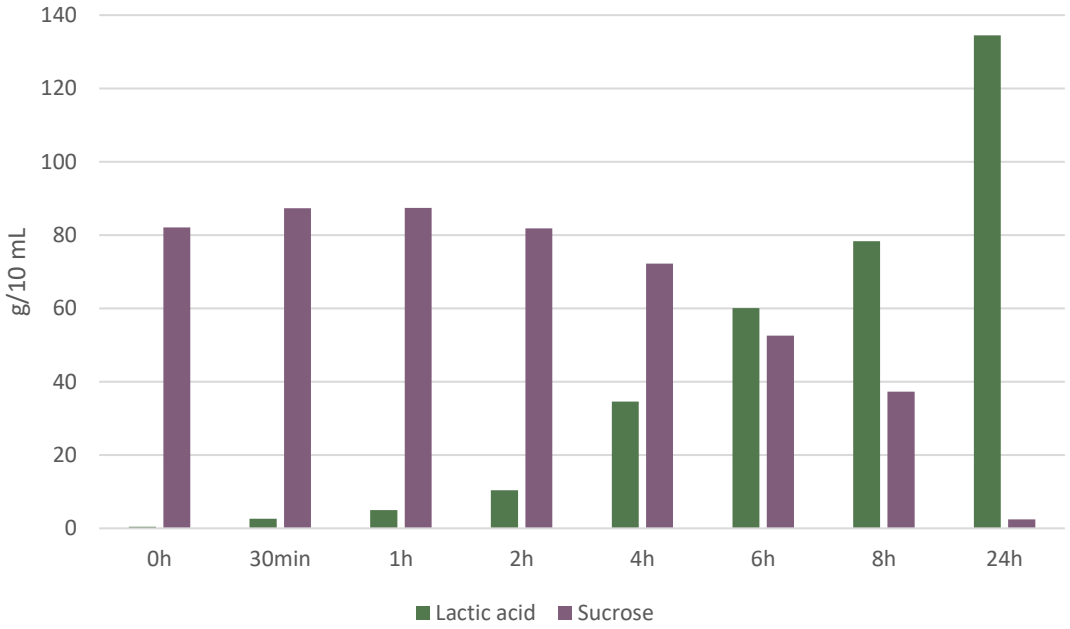

Figure 15 Mean of lactic acid and sucrose contents during fermentation with *Lbp. Plantarum* 10492.

Figure 16 shows the change in simple sugars and organic acids concentrations over time. In the graphs, it is evident how the sugar concentration decreases while the acidity level increases. The rise in acidity results from the changes in lactic acid concentration, and the decline in sugar concentration is a result of the reduction in sucrose and glucose. The pH decreases simultaneously with the increase in acidity, as expected.

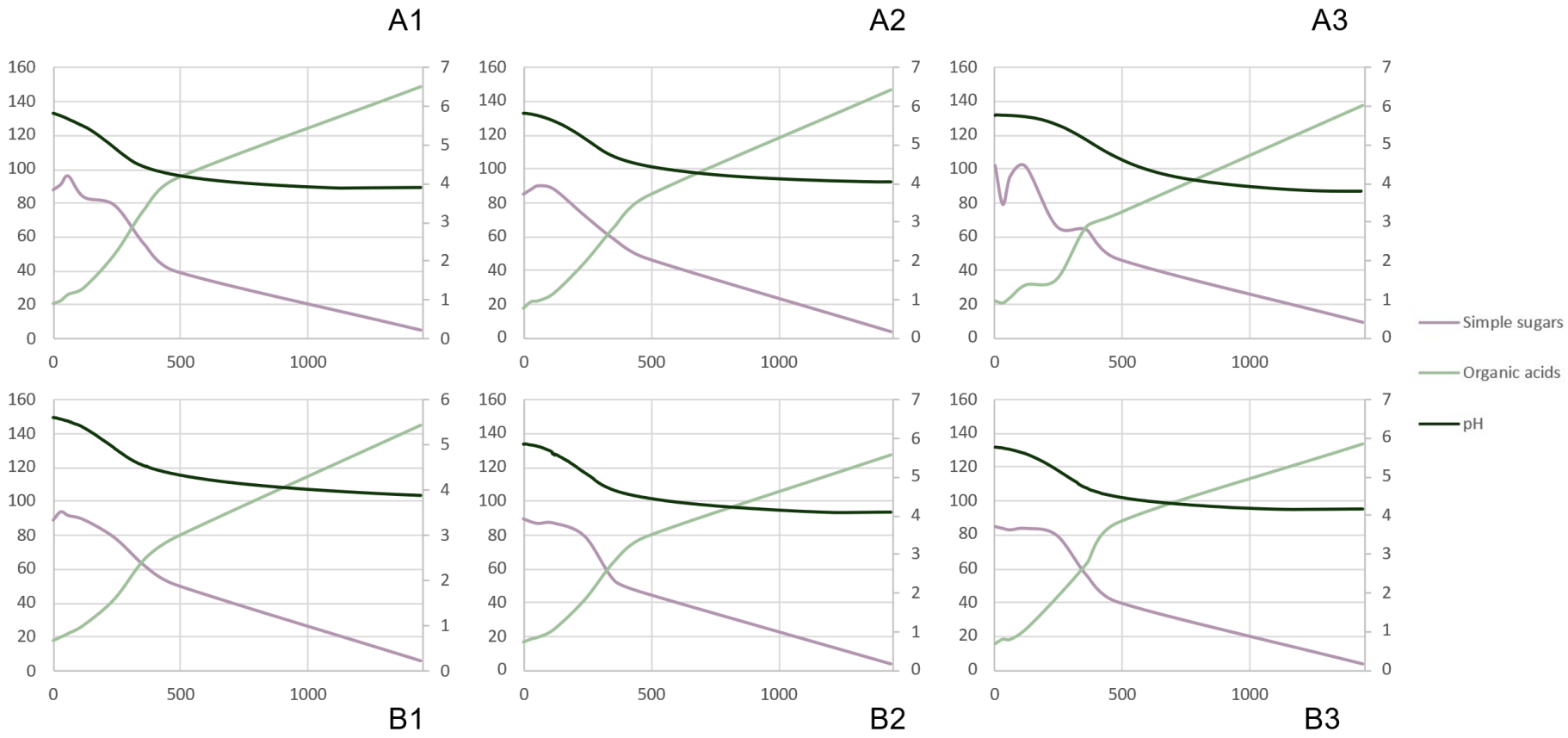

Figure 16 Simple sugars and organic acids content and pH during fermentation. The y-axis represents concentration (g/10 mL) or pH value, while the x-axis represents time (min). A samples were fermented with *Lpb. Plantarum* 10492 and B samples with *Lpb. Plantarum* 20174. Sugars are shown in lilac, organic acids in mint green and pH in dark green. Samples were collected from fermentation at 30 min, 1 h, 2 h, 4 h, 6 h, 8 h and 24 h.

The sugar content undergoes a slight initial increase at the onset of fermentation, followed by a subsequent decline. This phenomenon is likely attributed to the analysis focusing solely on simple sugars. Larger sugars undergo breakdown early in fermentation, as LAB hydrolyse polysaccharides.(Wang et al. 2021) Polysaccharides break down into simple sugars, which the GC-FIG method can be used to identify, leading to an apparent rise in the total sugar content concerning the identified sugars. Different bacteria strains may have varying effect on sugars.(Laaksonen et al. 2021)

The changes in pH occurred in a similar fashion across different fermentation batches. Before fermentation, the pH was 5.6 − 5.9 and during fermentation decreased approximately two units to 3.8 − 4.1. The changes in pH during fermentation closely resemble those reported in the literature (table 2). Prior studies have reported the pH reduction to be around two units.

3.3 Methodological considerations

The fermentation of lupin with the selected LAB was successful. The bacteria strains demonstrated their effectiveness in fermentation process and significant differences were observed between non-fermented and fermented samples. The LAB strains used in this study were from the same species and worked very similarly. More comprehensive understanding of lupin fermentation can be obtained by using a different species of LAB and studying how they perform lupin fermentations.

LAB fermentation for plant-based milk alternatives is used commercially to make plant-based cheese or yogurt alternatives. Commercial fermented plant-based alternatives have used many different plants as main ingredient. Oat, almond, and cashew are used for both yogurt and cheese alternatives whereas soy, pea, and coconut are fermented to make only yogurt. Fermentation can change the sensory and texture properties to mimic dairy products but still needs more research to understand more clearly fermentation affects plant-based foods. (Harper et al. 2022.)

Considering lupin´s nutritional profile and its benefits, particularly as a source of plant-based protein, it holds promising potential to be a future dairy alternative. With fermentation it could be used for example as alternative for cheese, yogurt or sour milk. However, before that, it would be beneficial to research lupin more, for example its digestibility and metabolic byproducts. Future lupin products need comprehensive sensory analysis and development for consumers to adapt lupin as dairy alternative.

4 Summary

The growing interest in lupins and their nutritional profile positions them as a potential novel plant protein source. Processing techniques can positively influence the nutritional attributes of lupins, and fermentation stands out as one promising method for lupin product processing. LAB fermentation has been extensively employed in dairy product processing, and research has indicated its potential applicability in the processing of plant-based milk substitutes.

Successful fermentation of lupin milk substitutes was achieved using a bioreactor with both LAB strains employed. The concentrations of glucose and sucrose decreased, while the lactate concentration increased as a consequence of fermentation. However, the fructose concentration remained unaffected. The concentrations of other organic acids experienced only marginal declines during fermentation.

Despite the promising prospects, there is limited research on lupin dairy alternative. Future investigations could explore the impact of fermentation on protein content, amino acid composition, or metabolic byproducts. Additionally, studying the influence of other LAB or alternative processing techniques could provide valuable insights.

Lupin products, particularly milk alternatives, hold the potential to thrive in future markets owing to their nutritional content and environmental sustainability.

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