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Use of emerging technologies to improve the solid-state fermentation of lupine seeds by *Pleurotus ostreatus*

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Abstract

Over the years, the increasing demand for animal proteins and, more broadly, the rise in meat consumption have stimulated interest in the development of plant-based protein alternatives. In this context, environmental and health-related aspects associated with intensive farming production systems have further contributed to strengthening the focus on more sustainable solutions.

The aim of this project is the implementation and optimization of existing techniques for the production of plant-based meat alternatives (PBMAs), specifically the solid-state fermentation of lupin seeds by *Pleurotus ostreatus*. Three different fermentation processes of lupin seeds were carried out under identical conditions of temperature, time, and humidity, while differing in the pre-treatment applied to the samples. Prior to sterilization and subsequent fermentation, one set of samples was treated with pulsed electric fields (PEF), another with high-power ultrasound (HPU) technology, while a third set was not subjected to any pre-treatment (CTRL). The objective of the project was to evaluate the impact of these different pre-treatments in order to assess their effectiveness and to consider the potential future implementation of these technological steps at an industrial level. All processes were conducted without the initial debittering step, in order to evaluate the influence of this choice on the subsequent aerobic fermentation.

The analyses carried out highlighted positive results associated with the application of these emerging technologies. These findings will need to be further investigated in the future, both through comparison with fermentation processes that include debittering and from the perspective of industrial and production-scale applicability.

Declaration of usage of generative AI and AI-assisted technologies

AI and AI-assisted technologies were used to revise some parts of the text and to identify relevant articles. One picture has been drawn with AI technology.

The author reviewed and edited the Thesis as needed and takes full responsibility for its content.

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Introduction

The global protein supply chain is constantly evolving, driven by population growth, the adoption of new technologies, and the increasing interest in personal health and wellness, which has now become a key objective for societies worldwide.

For many years, this growing demand for protein was met by expanding meat-based production, and for a long time, this approach was sufficient.

However, today this is no longer sufficient. The exponential increase in greenhouse gas emissions, largely driven by intensive livestock farming, forces us to seek new, more sustainable solutions that can both meet the growing demand and help reverse a trajectory that is already highly challenging from a climate perspective.

Alternative proteins have gained global attention as viable substitute for conventional animal-based foods. These include long utilized sources such as legumes, cereals, dairy, as well as emerging protein systems enabled by biotechnological innovation.

These products, known as plant-based meat alternatives (PBMAs), represent an important alternative to conventional meat and a significant opportunity for technological advancement. According to a global market forecast by MarketsAndMarkets, the PBMAs market is expected to reach USD 15.7 billion by 2027, with a compound annual growth rate (CAGR) of 14.7%.

Soy is currently the most widely used plant-based protein in the world, forming the basis of many meat alternatives due to its high protein content and versatile functionality. However, much of the global soy production is concentrated in regions far from Europe, often associated with deforestation and high environmental impacts. In contrast, lupin, a legume widely grown in the Mediterranean region, represents a promising local alternative. Rich in protein, sustainable to cultivate, and well-adapted to Mediterranean climates, lupin could become an important ingredient for plant-based products, reducing environmental footprints while supporting regional agriculture.

Fermentation of lupin seeds can help develop a possible alternative to soy-based products. Technological improvements in microbial fermentation can enhance the nutritional profile of lupin, increasing the availability of proteins and bioactive compounds, while simultaneously reducing antinutritional factors present in the seeds. This approach not only supports the production of high-quality plant-based ingredients but also takes in advantage local Mediterranean crops in a more sustainable way.

This dissertation has been made during an Erasmus program at the he Polytechnic University of Valencia shortened to UPV, under the supervision of Prof. José Vicente Garcia Perez.

The aim of this study is to evaluate the outcomes of solid-state fermentation of lupin seeds using the fungus Pleurotus ostreatus, focusing on protein content and antinutritional compounds. The process was investigated both without pretreatment (CTRL) and with technological pretreatments, such as pulsed electric fields (PEF) and high-power ultrasounds (HPU). Fermentation was monitored over a 14-day period, starting from day 0, with samples collected every two days to consistently evaluates fungal growth and the associated changes in the seeds nutritional and antinutritional profiles.

Furthermore, this study has been applied considering a debittering-free approach, in the way to evaluate the effectiveness of fermentation process without this known pretreatment. The primary purpose of the debittering process in lupin seed fermentation is to remove bitter-tasting alkaloids, which could compromise both food safety and the quality of the final product. However, this pretreatment may also strip away important nutrients that are beneficial for fermentation and microbial development. Therefore, exploring a fermentation approach without debittering could be advantageous, as it preserves these essential compounds, potentially enhancing fungal or bacterial growth and improving the overall nutritional and functional properties of the final product.

The first chapter addresses the growing need to improve these PBMAs as a substitute of meat. Furthermore, it provides an overview of this new potential market.

Second one provides a more technical discussion of the solid-state fermentation process, including the rationale for selecting *Pleurotus ostreatus* as the microorganism and the adoption of a debittering-free approach. Additionally, the chapter introduces the two technological strategies aimed at enhancing fermentation performance.

The third chapter describes the materials and methodology used in this project. It also details the analytical protocols employed to assess the nutritional and antinutritional compounds. Furthermore, the chapter evaluates the impact of fermentation on the techno-functional properties of the lupin seeds.

The fourth chapter presents the results obtained and provides a comparison, assessing the effects of the different technological pretreatments.

Some final remarks and a discussion on future possible developments will conclude the work.

Chapter 1
Rationale for plant-based protein alternatives

This chapter focuses on the reasons why further improvements in technologies related to PBMAs are necessary in the coming years. It highlights global meat consumption trends and the issues associated to these productions: the consistent amount of global greenhouse emissions and the antimicrobial resistance in food production are the most relevant ones. Furthermore, it provides a narrative review of the main protein sources alternatives, and it focuses on the possibilities of PBMAs.

1.1 Global Trends in Meat and Animal Protein Consumption

Over the past century, meat and animal protein consumption has increased significantly, playing a central role in the transformation of the global food system. This growth reflects broader structural changes, including rising per capita incomes, urbanization, and shifting dietary patterns toward an increase of animal proteins consume.

Proteins are composed of amino acids, nine of which are indispensable and must be obtained through the diet. The nutritional quality of dietary proteins is affected by the type of amino acids they contain as well as the level of digestibility. The FAO recommends evaluating protein quality using the Digestible Indispensable Amino Acid Score (DIAAS) (FAO, 2013; Leser, 2013), which assesses the digestibility of individual indispensable amino acids at the end of the small intestine and provides a detailed measure of how effectively a protein source meets human requirements. In this context, animal proteins tend to exhibit high quality due to their well-balanced amino acid profile and high digestibility. In particular, meat proteins are considered among the highest-quality dietary sources, as they supply all indispensable amino acids in proportions close to human needs and are efficiently utilized by the body.

Regarding the historical trend of per capita meat consumption, the most pronounced increase was observed between the mid-1960s and the late 1990s. According to a report by FAO (2003, as cited in Heinrich Böll Foundation & Friends of the Earth Europe, 2014), global per capita consumption rose from 24.2 kg in the period 1964–66 to over 36 kg in 1997–99, highlighting a clear upward trend in meat consumption at a global scale.

In subsequent years, this trend showed a slowdown, with an average of approximately 39.5 kg in 2010 and around 42–43 kg in 2020, indicating that the growth of per capita consumption worldwide has become more moderate compared to recent decades.

However, these aggregated global values conceal significant disparities between developed and developing countries. In industrialized nations, per capita meat consumption has traditionally been much higher: in the past, average values exceeding 80–90 kg per year were common in many high-income economies, whereas in developing regions, levels remain significantly lower, often ranging between 20–30 kg per capita per year, or even less in many areas of sub-Saharan Africa and South Asia. Today, developed countries show a tendency toward saturation or stabilization of per capita consumption, with very limited increases or even stagnation, whereas in developing countries, the rise in per capita meat consumption is still ongoing, driven by increasing incomes, urban population growth, and changes in dietary habits. According to FAO databases, the increase in meat consumption in developed countries was only 3.2% between 2010 and 2020, confirming the saturated situation, while it exceeded 16% in developing countries.

Over the past decades, the composition of global meat production has undergone a profound transformation. Considering the recent Food Balance Sheets (FAO,2022), poultry has consistently been the fastest-growing category, increasing its share of total meat production from around 30% in 2000 to approximately 36-38% in the early 2020s, overtaking pork as the leading meat type worldwide. Pork, which accounted for nearly 38–39% of global meat production around 2000, has seen its relative share decline to about 34%, despite continued growth in absolute volumes. Beef production has also experienced a relative contraction, with its share decreasing from roughly 24% at the turn of the century to around 19–20% today, while mutton and goat meat have remained a minor but stable component of total output. These shifts reflect the higher efficiency, lower production costs and shorter production cycles of poultry, and to a lesser extent pig production, in converting feed into edible protein, compared with bovine and small ruminant species. As a result, growth in global meat supply has been increasingly driven by the expansion of poultry and pig production systems, while per capita consumption of ruminant meat has stagnated or declined in many high-income regions.

Recent projections indicate that global meat demand growth is slowing compared with the rapid expansion observed in the late twentieth century, reflecting market saturation in many high-income countries and a deceleration of global population growth. Although the growth of global per capita meat consumption has slowed, particularly in high-income countries where consumption levels are already high, total meat demand continues to rise due to global population growth and urbanization, especially in developing regions. This means that even with moderate increases in per capita intake, the absolute volume of meat produced and consumed is still expanding, maintaining pressure on natural resources, greenhouse gas emissions, and environmental sustainability.

In conclusion, global meat and animal protein consumption continue to increase in absolute terms but show relative deceleration, while plant-based proteins continue to play an essential complementary role, particularly through legumes and cereals consumed in combination.

PBMAs are emerging as crucial tools to address environmental and nutritional constraints in global food systems, allowing for a more sustainable fulfilment of global protein requirements.

The integration of animal and plant proteins thus represents a key strategy to ensure protein security, nutritional balance, and long-term environmental sustainability.

1.2 The need for plant-based alternatives

The steady increase in global meat production over the years has led to a range of issues that are now evident to everyone: significant greenhouse gas emissions, the rise of antimicrobial resistance linked to food production, and poor animal welfare conditions in intensive livestock farming. For these reasons, it is necessary to identify more sustainable alternatives, such as PBMAs.

1.2.1 Global greenhouse gas emissions from food production

Based on global meat production data, the importance and the considerations on the implications of this dietary model and the need to develop PBMAs become evident. Although the growing production of meat is supported by production efficiency and technological innovations, it exerts significant pressures on natural resources, greenhouse gas emissions, and the sustainability of global food systems.

The study by Xu *et al.* (2021) enabled the assessment and quantification of CO_2, CH_4, and N_2O emissions associated with the production and consumption of all plant- and animal-based foods in circa 2010. This analysis considered all stages of agricultural land management as well as emissions from feed production on croplands and pastures, using a balance between raw materials produced and consumed. In this study, livestock emissions included only those resulting from enteric fermentation and manure management, as quantified according to the Intergovernmental Panel on Climate Change (IPCC) and the Food and Agriculture Organization of the United Nations (FAO).

Regarding the food production, total global greenhouse gas emissions associated with food systems, including croplands, livestock, and land-use change (LUC), amounted to $17{,}318 \pm 1{,}675\ Tg\ CO_2\, eq$ year^{-1} (Xu *et al.* 2021). It is important to distinguish this quantity of emissions according to per capita rates, emissions per unit of land area, and the contribution of animal- or plant-based production. For example, South and Southeast Asia (SSEA) accounted for the highest share of food-related emissions (23%); however, it exhibited low per capita emissions and was the only region in which emissions from plant-based food production exceed those from animal-based sources.

Considering the different greenhouse emissions by food source, plant-based production accounted for 29% of total food-related emissions (19% CO_2, 6% CH_4, and 4% N_2O), whereas emissions derived from animal-based production accounted for 57% of total emissions, consisting of 30% CO_2, 20% CH_4, and 7% N_2O. This calculation included emissions from croplands (8%) and pastures (13%) used for feed production, emissions from livestock farming (20%), which were mainly attributable to enteric fermentation in ruminants (18%) and manure management (2%), emissions associated with land-use change LUC (12%), and post-production emissions such as transport and processing, which contribute 4% of the total.

According to the study, the regions with the highest greenhouse gas emissions from animal-based food production included South America, accounting for 14% of all global food-related emissions (in particular Brazil, which alone accounted for 6% of global emissions due to deforestation-related to LUC), followed by China with 8% of total emissions, the United States with 5%, and India with 4% of the global total. The European Union was finally responsible for a substantial share of greenhouse gas emissions associated with both imports and exports of animal-based foods, mainly due to the high volume of international trade.

Considering the study by Poore and Nemecek (2018), it was also possible to compare the average impact in terms of kilograms of CO_2-equivalent emissions relative to the protein content of different food products. This relationship between greenhouse gas emissions and protein production highlighted a profound imbalance between the resources employed and the nutritional output obtained, explaining why the choice of protein source is a key determinant of environmental footprint. It has been observed that animal-based products (including meat, aquaculture, eggs, and dairy) use approximately 83% of global agricultural land and contribute around 56–58% of total food-related emissions, while providing only 37% of global protein supply and 18% of total calories consumed worldwide. A direct comparison between CO_2eq emissions per 100 grams of protein from animal- and plant-based sources is presented in the following table 1.1.

Table 1.1. *Greenhouse gas emissions associated with different food sources*

Food source	Mean Value [kg CO_2eq]	10th Percentile [kg CO_2eq]
Beef (beef herd)	49.9	20
Lamb & Mutton	19.9	12
Beef (dairy herd)	16.5	\$9.
Pig Meat	7.6	4.6
Poultry Meat	5.7	2.4
Grains	2.7	1.0
Tofu	2.0	1.0
Groundnuts	1.2	0.6
Peas	0.4	0.3

As shown in the Table 1.1, there is a substantial difference between the emissions generated by animal-based sources and those from plant-based sources when compared on an equal protein-content basis. Even the 10th percentile of the lowest-emitting animal products (such as poultry) produces significantly higher greenhouse gas emissions than the average of most plant-based foods.

Another key aspect addressed in this study is the inefficiency of protein conversion in the animal-based systems analysed. According to Xu *et al.* (2021) only 8.49% of the protein contained in feed provided to livestock is effectively converted into edible protein for human consumption; this implies that more than 91% of the plant-derived protein used to feed animals is lost during the biological conversion process.

1.2.2 Antimicriobial resistance in food production

Another important aspect to consider is the correlation between the extensive use of antimicrobials in intensive livestock farming and the increasing amount of pathogens resistant to these compounds. According to Thomas P. Van Boeckel *et al.* (2019), the growing global demand for meat and animal-based proteins is driving an increasing use of antibiotics in livestock production, with approximately 73% of all antimicrobials sold globally being used in food-producing animals, and, in parallel, a rise in antimicrobial resistance among microbial pathogens. Beyond the potentially severe consequences for public health, this strong dependence on antimicrobials represents a threat to the long-term sustainability of the livestock sector and to the security of global food systems.

Focusing on low- and middle-income countries (LMICs) and considering common indicator pathogens such as *Escherichia coli*, *Campylobacter* spp., non-typhoidal *Salmonella* spp., and *Staphylococcus aureus*, the study showed that between 2000 and 2018 the proportion of antimicrobial compounds with resistance levels exceeding 50% (P50) increased from 0.15 to 0.41 in poultry and from 0.13 to 0.34 in pigs, while remaining between 0.12 and 0.23 in cattle. These interspecies differences were consistent with varying degrees of production system intensification, which were more pronounced in poultry and pig farming than in cattle production. The regions where the highest number of resistance hotspots was observed included northeastern India, northeastern China, northern Pakistan, Iran, eastern Turkey, the southern coast of Brazil, Egypt, and the areas surrounding Mexico City and Johannesburg, highlighting a geographically heterogeneous but strongly clustered distribution.

This particularly rapid increase has been observed mainly in developing countries rather than in high-income countries, primarily due to lower levels of biosecurity, less nutritious feed, and more permissive regulations governing the use of veterinary drugs. In addition, it’s important to highlight the limited presence of structured monitoring and surveillance systems, which makes the early identification of resistance hotspots more difficult. The study also placed particular emphasis on antimicrobials classified as “critically important” for human medicine by the World Health Organization, as their loss of efficacy in animals can significantly reduce the therapeutic options available for treating human infections. Specifically, the highest resistance rates were observed for *ciprofloxacin* and *erythromycin* (20–60%), while moderate rates were recorded for third- and fourth-generation *cephalosporins* (10–40%).

Overall, these findings constitute a major global warning, as the range of antimicrobials used in the production of food animals is rapidly diminishing, with serious implications for both animal and potentially human health. Due to the global nature of antimicrobial resistance, this issue cannot be managed only at the local level and requires coordinated international strategies. It is therefore crucial to reduce the rise of antimicrobial resistance by promoting a transition to more sustainable livestock practices and by supporting the development and adoption of alternative foods, such as plant-based proteins, which can help reduce reliance on conventional meat production.

1.2.3 Intensive farming and ethical considerations

It is now important to address an ethical issue related to the global increase in meat and animal protein consumption: intensive livestock farming. This method involves confining animals in restricted spaces, with the primary goal of maximizing the production of meat, milk, or eggs. From a legal perspective, the concept of Concentrated Animal Feeding Operations (CAFOs) has been defined by the United States Department of Agriculture (USDA,2020) as an intensive animal feeding operation (AFO) in which over 1,000 animal units are confined for over 45 days a year (EPA,2012). One animal unit corresponds to 1,000 pounds of live animal weight, and the thresholds defining these categories vary depending on the type of livestock operation considered. By 2023, it was estimated that about 74% of all terrestrial livestock lived in intensive or industrialized farming systems (Our World in Data, 2024).

CAFOs have raised numerous ethical and environmental concerns. In terms of animal welfare, restricted space, overcrowding, and lack of access to shelter and natural environments can cause significant suffering, disease, and chronic stress. This form of agriculture relies on particularly aggressive confinement methods, in which animals are forced to live in densely crowded, stimulus-deprived environments. Key concerns associated with this farming system include animal mutilation, often carried out to reduce aggression; selective breeding aimed at enhancing productivity; the occurrence of diseases, partly resulting from reduced genetic diversity; the preventive use of antibiotics, as mentioned in the previous paragraph, and chronic stress arising from the restrictive living conditions (Singer, 1975; Rollin, 2006).

Regarding human health risks, intensive livestock operations, particularly CAFOs, not only contribute to greenhouse gas emissions but also represent a significant source of negative impacts on water and air quality, mainly due to the large volumes of animal waste generated (Walker et al., 2005).

From a hydrological perspective, surface waters can be contaminated by CAFOs waste through the discharge of nutrients, organic matter, and pathogens from fields and storage facilities. These pollutants can subsequently leach into groundwater. Such waste contains various potentially harmful contaminants, including nitrogen and phosphorus, pathogens, trace elements such as arsenic, pesticides, and hormones. Consequently, these large quantities of waste lead a high risk to water quality and water ecosystems.

Similarly, air quality can be affected by the release of gases harmful to humans, such as ammonia, sulfuric acid, methane, and particulate matter. In particular, the Intergovernmental Panel on Climate Change (IPCC,2019) recognizes the substantial contribution of livestock to methane emissions and recommends reducing sources of chronic stress and modifying animal feeding practices to mitigate these harmful emissions.

The high global demand for meat and animal-based proteins, combined with the increasing prevalence of intensive livestock farming worldwide, has created significant ethical concerns regarding animal living conditions, as well as environmental impacts, ultimately affecting human health. Therefore, it is essential to implement sustainable alternatives capable of replacing and satisfying the current demand for animal-derived protein.

1.3 Alternative Protein Sources: Overview, Applications and Market Potential

In the context of the growing focus on more sustainable food systems, the alternative protein sector plays a key role. Specifically, three main categories of alternatives to conventional meat can be identified: plant-based proteins, insect-derived proteins, and cultivated meat.

Cultivated meat is produced through the proliferation and differentiation of animal muscle cells in bioreactors under controlled conditions, with the aim of replicating the characteristics of meat obtained through traditional livestock farming (Post *et al.*, 2020). However, cultivated meat currently faces numerous challenges, including extremely high production costs, technical difficulties in replicating complex cuts, and a regulatory framework that is still under development.

Insect-derived proteins, on the other hand, offer a complete amino acid profile and high concentrations of micronutrients, such as iron and zinc. Despite these nutritional advantages, their commercial development remains limited, largely due to significant psychological and cultural barriers that inhibit consumer acceptance (Hartmann *et al.*, 2015).

Today the most viable and widely adopted solution appears to be plant-based proteins, and particularly PBMAs, which already benefit from a relatively mature market and represent a valid option in terms of environmental sustainability.

1.3.1 Historical Development of Plant-Based Meat Alternatives: a Narrative Review

To contextualize PBMAs sector, it is useful to briefly trace its origins, an area that, although often perceived as recent, actually has roots in dietary practices developed over the centuries. Production of an early form of seitan is documented as far back as around 600 CE (Shurtleff W., & Aoyagi, A., 2014), while wheat- and soy-based analogues only began to be commercially available in the early twentieth century. From the second half of the twentieth century, advances in food technologies facilitated the emergence of a new generation of products, characterized by increasing attention to the structural and functional properties of plant proteins (Kyria et al., 2021).

Taking in account the case of soy proteins, Zhang *et al.* (2021) noted that these products gained popularity in Western markets as meat alternatives only in the early 1960s. However, in Asia, soy has been used for centuries in the production of traditional protein-rich foods such as tofu, tempeh, yuba, and tofu skin, representing a long-established component of the daily diet CE (Shurtleff W., & Aoyagi, A., 2013).

In addition to cereal-based proteins and soy, further historical examples of PBMAs can be identified in the use of microorganisms and fungi as protein sources. Traditional fermentation processes, employed for centuries across different regions of the world, enabled the production of foods with high nutritional value, conceptually anticipating some of the principles bases of modern food engineering. From the second half of the twentieth century onward, this body of knowledge was progressively translated into industrial applications, leading to the experimentation and adoption of fungal proteins as alternative meat ingredients (Finnigan, T. J. *et al.,*2018)

Alongside these technological developments, social and cultural dynamics also played a crucial role in shaping the emergence of meat alternatives. In Europe and North America, particularly between the nineteenth and early twentieth centuries, vegetarian and reformist movements actively promoted the development and consumption of early plant-based meat analogues, often driven by ethical, religious, and health-related considerations. These movements not only influenced individual dietary choices but also contributed to the construction of a broader cultural and normative framework that supported research, innovation, and consumer acceptance of alternatives to conventional meat.

Together, these historical experiences and the advancement of food processing technologies helped shape the cultural and scientific bases upon which the contemporary PBMAs sector has developed.

1.3.2 Plant-Based Meat Alternatives: Key Sources, Processing Technologies and Global Market Trends

Over the past decades, the PBMAs sector has experienced substantial growth, establishing itself as one of the most dynamic and ambitious areas of the global food industry. This expansion has been primarily driven by increasing concerns about environmental sustainability, shifts in consumer dietary habits, and a growing interest in alternative dietary models that reduce or eliminate the consumption of animal-derived meat. Within this context, PBMAs now represent a well-established product category, characterized by a wide range of offerings and an increasingly diverse use of plant-based protein sources. This sector is gaining widespread popularity and consumer acceptance, particularly among younger generations, who are increasingly prioritizing environmental impact and animal welfare in their food choices.

The main raw materials used in the formulation of plant-based meat alternatives include legumes, cereals, and pseudocereals, selected based on their protein content, functional properties, and large-scale availability. Historically, soy has been the most widely used plant source for this type of production (Zhang *et al.*,2021); this predominance is largely attributable to both the widespread availability of the raw material and the techno-functional characteristics of soy proteins, including their solubility, water- and oil-absorption capacity, as well as their gelling and emulsifying properties. These attributes play a crucial role in determining the quality of the final product. However, research and market interest are progressively shifting toward soy alternatives, driven by concerns related to allergenicity, the vulnerability of soy cultivation to climate-related impacts, and the need to preserve biodiversity. Consequently, recent studies have explored the potential of proteins derived from peas, faba beans, rapeseed, lupins, and hemp, used either individually or in hybrid formulations in combination with soy.

The geographical distribution of major plant-based protein sources largely reflects global agricultural and trade dynamics. Soybean cultivation, for instance, is predominantly located outside Europe, with production highly concentrated in North and South America. This production pattern results in a significant dependency on imports for many European countries (Lassaletta *et al.,* 2014), representing a critical issue particularly in the Mediterranean region, where climatic conditions are not always suitable for large-scale soybean cultivation. Consequently, recent years have seen growing interest in alternative plant-based protein sources better adapted to local agricultural contexts. These sources have the potential to reduce dependence on imports while enhancing the valorisation of regional resources, thus contributing to open new opportunities for the development of more sustainable and resilient protein supply chains.

According to Andreani *et al.,* Nutrients (2023), early formulations of PBMAs relied predominantly on protein isolates, rather than flours or whole seeds, with protein contents exceeding 75% and often approaching 90%. However, the production of protein isolates requires wet separation processes that are frequently time-consuming, costly, and inefficient, due to the extensive use of water, acids, or enzymes. In response to these limitations, the sector has increasingly shifted toward the use of protein concentrates, which typically contain between 50% and 65% protein. These ingredients provide the structural properties required in the final product and are easier to obtain; indeed, protein concentrates are produced through dry fractionation techniques, which use less water and energy and better preserve the native protein structure Andreani *et al.,* Nutrients (2023).

Starting from the raw material, a meat-like structure is achieved when the native globular structure of legume proteins is transformed into a fibrous arrangement, in which the proteins are elongated and highly ordered (Andreani *et al.,* Nutrients (2023)). This restructuring process converts globular aggregates into an anisotropic matrix composed of elongated protein polymers stabilized by new intermolecular bonds. Such a structure can be produced using various technologies, among which extrusion is by far the most widely employed. During extrusion, the raw materials are hydrated and subjected to mechanical and thermal stresses, which break the weak bonds that maintain the protein's native globular structure. The resulting protein paste is then forced through an opening, following the physical constraints of the process, which aligns the now-elongated protein molecules parallel to the flow direction. Depending on the moisture content, extrusion can produce textured vegetable proteins (TVP) at low moisture levels (<30%) or directly create meat analogues at high moisture levels (>50%). The high productivity, low cost, versatility, energy efficiency, and scalability potential of extrusion have made it the dominant technology for producing meat analogues (Andreani *et al.,* Nutrients (2023)). Emerging techniques, such as high-moisture shear cell processing and additive manufacturing live 3D printing (Xavier *et al.,* 2025) offer promising alternatives to extrusion; however, their industrial scalability still requires optimization of production costs and greater versatility in processing different plant protein sources.

In recent years, the global market for plant-based meat alternatives has experienced significant growth, driven by increasing consumer interest and evolving dynamics within the food sector. Estimates indicate that the global market for meat alternatives is expected to reach approximately USD 8.1 billion by 2026, with a compound annual growth rate (CAGR) of 7.8% over the period 2019–2026 (Vallikkadan *et al.,* 2023). This expansion can be attributed to several factors, including a higher consumer awareness of potential health risks associated with high consumption of traditional meat, as well as growing attention to ensuring adequate protein intake in modern diets.

From a geographical perspective (Xavier *et al.,* 2025), Europe currently holds the largest share of the global market for meat alternatives, accounting for 51.5%, followed by North America (26.8%), the Asia-Pacific region (11.8%), Latin America (6.3%), and the Middle East and Africa (3.6%) (Vallikkadan *et al*., 2023). Despite Europe's market leadership in terms of share, studies on consumer perceptions indicate particularly strong interest in Asian countries. A survey conducted in the United States, China, and India revealed that the proportion of consumers favourable toward meat alternatives is significantly higher in China (95.6%) and India (94.5%) compared to the United States (74.7%) (Bryant *et al.*, 2019), suggesting substantial potential for future market growth in these regions.

Despite its rapid development, the market for plant-based meat alternatives remains relatively small compared to the overall meat market, highlighting that the sector is still in a consolidation phase. Nonetheless, several studies indicate that the PBMAs sector is expected to grow at an accelerated pace in the coming years, positioning it well for further expansion and innovation.

Chapter 2
Case study: Lupine seeds and Fermentation with Pleurotus Ostreatus

This chapter presents the case study. Firstly, lupin seeds are introduced and the reasons supporting their suitability as a raw material for PBMAs are outlined. Subsequently, the fermentation process is outlined, with particular emphasis on the solid-state fermentation (SSF) approach adopted during the experimental phase. The characteristics of the selected fungal strain, *Pleurotus ostreatus*, are then described, together with the rationale behind its selection. Finally, the technological pre-treatments applied to optimize the fermentation process are presented, namely pulsed electric fields (PEF) and high-power ultrasound (HPU).

2.1 Lupine seeds: Characteristics, properties and explanation of the choice

Lupins are an important family of legumes in nature due to their ability to produce seeds rich in proteins, dietary fiber, and mono- and polyunsaturated fatty acids. Moreover, they are considered an important resource because of their capacity to tolerate adverse growing conditions, demonstrating a high degree of geographical and environmental adaptability. Lupin species have been found in Europe, Africa, and the Americas, where they have readily adapted to a wide range of climatic conditions. According to Knecht *et al.* (2020), the earliest evidence of domesticated lupin seeds comes from Egyptian tombs dating back to the 22nd century BC, testifying to their already established use in ancient times. Subsequently, the use of lupin seeds gradually spread to other areas of the Mediterranean basin. Evidence of the presence of lupin in the New World, on the other hand, dates to the 6th–7th century BC.

Lupins are large plants with flowers that can be blue, yellow, or white. The seeds of these plants, particularly those of the species *Lupinus albus*, *Lupinus luteus*, *Lupinus angustifolius*, and *Lupinus mutabilis* (Johnson *et al.,* 2017), have long been used as a food source for both livestock and humans, as well as a means of enriching the soil. The ability of this legume family to adapt to different soil types and to grow under drier conditions than soybean, combined with their lower requirements for nitrogen and phosphate fertilizers to achieve significant yields, makes them a particularly interesting and increasingly cultivated alternative. This sector, after reaching its production peak in the 1990s with approximately 2.1 million tons in 1999, has shown some variability over the years, before recently stabilizing at around 1.60 million tons globally (FAOSTAT, 2022).

Considering the growing interest in the field of food engineering and plant-based alternatives, this value is approximately 51% higher than production ten years earlier and represents an increase of 13.8% compared to the previous year, confirming a renewed dynamism in the sector. Nutritionally, lupin seeds are distinguished among legumes by both their protein and dietary fiber content. The kernel (the inner part of the seed) contains between 37 and 40 g of dietary fiber per 100 g of dry weight (Johnson *et al.,* 2017). In terms of protein, lupin seeds provide around 40 g per 100 g of dry weight, representing the highest protein content of any commonly consumed legume (Mülayim *et al*., 2002). Moreover, this protein exhibits a high digestibility, approximately 98% (Chew *et al*., 2003), comparable to that of soybean. The combination of high fiber and protein content contributes to beneficial effects on lipid metabolism, including the reduction of LDL cholesterol and overall improvement of the lipid profile, thereby supporting cardiovascular health.

Nevertheless, the use of these seeds as food remains currently limited due to the presence of variable amounts of anti-nutritional compounds, such as alkaloids, protease inhibitors, lectins, and saponins, which may have adverse effects on human health. In particular, the alkaloids present, mainly belonging to the quinolizidine family, are of significant concern due to their potential toxicity to humans. For this reason, their maximum legal level has been set at 0.02 g per 100 g in lupin flour and lupin-based products by several authorities, including those in Australia, New Zealand, the United Kingdom, and France (Food Standards Australia New Zealand, 2016b).

In conclusion, lupins represent an interesting and promising option for the future of PBMAs, due to their high protein and fiber content, and the rich nutritional profile. However, they still present challenges related to anti-nutritional components, which need to be addressed as much as possible to enable the full development of the sector. These issues can be partially mitigated through fermentation, which promotes the biotransformation of anti-nutritional compounds and can increase the content of bioactive metabolites, thereby enhancing both the safety and the nutritional value of the final product. Lupin is highly suitable for this type of treatment, serving as an ideal substrate for the growth and metabolic activity of fermentative microorganisms.

For the experimental part of this case study, the species *Lupinus albus* was selected, mainly due to its high protein content, low alkaloid levels in “sweet” varieties, and wide commercial availability, making it particularly suitable for both fermentation studies and the development of high-quality plant-based food products.

2.2 Solid-State Fermentation (SSF): Principles and Applications

Fermentation is a biotechnological process in which microorganisms such as bacteria, yeasts, and fungi transform organic substrates through their metabolic activity, producing compounds of nutritional, functional, or technological interest. It can occur in a liquid state (SmF, submerged fermentation), where microorganisms grow in an aqueous medium containing nutrients and dissolved gases, a condition widely used at the industrial level for precise control of process parameters. Alternatively, fermentation can take place in a solid state (SSF, solid-state fermentation), in which microorganisms develop on solid substrates with a minimal amount of free water, in an environment dominated by a gaseous phase. This one represents an interesting industrial alternative, as it is particularly suitable for the valorisation of agro-industrial residues and to produce enzymes and bio compounds, offering advantages such as higher product concentration and lower water and energy consumption.

Since the 1960s, after years of predominant development in submerged fermentation, SSF has regained the interest of both scientists and industry due to its ability to ferment low-cost substrates such as agro-industrial residues, representing an environmentally friendly solution for the treatment of polluting solid waste (Mitchell *et al.,* 2006). According to Garrido-Galand *et al.* (2021), the combination of practical, economic, and environmental advantages offered by SSF compared to SmF has played a key role in this growing attention. These advantages include: higher final product concentrations, due to reduced substrate inhibition (with conversions of 20–30% in solid fermentation compared to about 5% in liquid fermentation); lower water consumption and reduced water activity, making the system less susceptible to contamination; higher volumetric productivity, thanks to the use of more compact bioreactors; and simpler, more cost-effective product extraction processes. Another significant aspect concerns the inoculum: SSF is particularly suitable for the growth of filamentous fungi, as the operational conditions more like the natural environment to which these microorganisms are evolutionarily adapted.

For these reasons, the case study employed this type of fermentation, which is particularly suitable for the chosen fungal species, *Pleurotus ostreatus*.

Regarding PBMAs production, SSF represents a valid alternative, an integration, and potentially a more efficient process compared to current conventional methods for producing meat analogues. According to Milcarz & Harasym (2025), unlike conventional methods, which are primarily based on physical processes such as extrusion and high-temperature, high-pressure treatments, SSF relies on a biological approach that exploits the metabolic activity of microorganisms under ambient temperature conditions. Nutritionally, conventional processes tend to preserve the natural protein content with limited improvements, whereas SSF can enhance nutritional quality through the reduction of anti-nutritional factors and the improvement of digestibility. Finally, in terms of sustainability, conventional methods generally require higher energy input and make limited use of by-products, while SSF requires less energy and allows the valorisation of agro-industrial residues, making it a more sustainable and efficient alternative.

The defining feature of SSF is the growth of microorganisms on water-insoluble substrates, maintained at a moisture level sufficient to support microbial metabolism but below the water-holding capacity of the solid matrix. This condition results in the formation of a three-phase system, as illustrated in Figure 1.1, consisting of solid substrate particles, a thin liquid film adhering to their surface, and gas-filled pores that allow oxygen transfer. This configuration often leads to higher productivity and distinct metabolic profiles compared to submerged fermentation (Milcarz & Harasym, 2025).

In SmF, oxygen must diffuse from the gas phase into the liquid, whose density and low oxygen solubility require intensive agitation and aeration to ensure adequate oxygen availability. In contrast, in SSF, the absence of a dense liquid phase allows microorganisms to directly exploit the more oxygenated porous zones, improving not only local oxygen supply but also nutrient transfer and metabolite removal, making the process overall more efficient in terms of mass transfer.

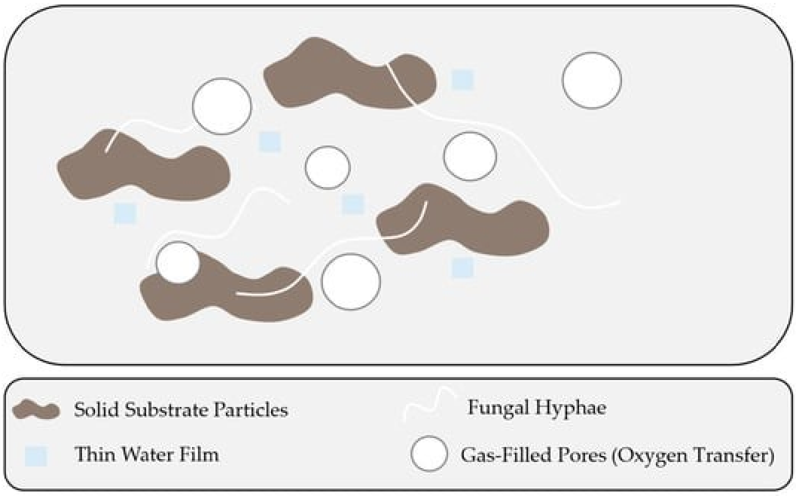


Fig 1.1: *Milcarz, A., & Harasym, J., 2025*
Three-phase structure of solid-state fermentation

However, significant challenges still arise in the scale-up of SSF. Unlike SmF, where scaling follows well-established engineering principles, the heterogeneous nature of SSF and the difficulties in removing heat from large substrate beds greatly complicate industrial design (Milcarz & Harasym, 2025). Although oxygen transfer is locally efficient, it is difficult at a large scale to ensure that all regions of the substrate receive sufficient oxygen and maintain optimal moisture. This can lead to anoxic or overly dry micro zones, reducing overall productivity. The heterogeneity of the substrate, which varies in density, particle size, and chemical composition, further complicates the uniform control of pH, nutrients, and physical conditions throughout the fermentation bed. From a thermal perspective, in large substrate masses, the heat generated by microorganisms accumulates rapidly due to the low thermal conductivity of the solid material, creating temperature gradients that can impair microbial growth and alter metabolite production.

Despite these challenges, advances in bioreactor design, process monitoring, and control strategies are significantly improving the industrial feasibility of SSF processes, making this technology an important resource in food engineering for the production of new PBMAs.

2.3 Fungal fermentation: the role of *Pleurotus ostreatus*

For the SSF process, the fungal species selected for metabolic activity is the white-rot fungus *Pleurotus ostreatus*, one of the most well-known edible fungi worldwide and classified as GRAS (Generally Recognized As Safe) by the Food and drugs Administration (FDA). In addition to its availability and food-grade suitability, this species is already employed industrially, particularly to produce enzymes such as laccases and cellulolytic enzymes, as well as for the biotransformation of agro-industrial by-products, including lignocellulosic residues, protein-rich biomass, and feedstocks with enhanced nutritional value.

This species is particularly well-suited to SSF because the technique replicates the fungus's natural growth conditions: a solid environment, controlled but not excessive moisture, and limited availability of easily accessible nutrients. The fungus's filamentous mycelial structure enables efficient three-dimensional colonization and the secretion of extracellular enzymes directly into the matrix, facilitating biotransformation processes without the need for a free liquid phase.

Beyond its well-known industrial applications, the fermentation of P. *ostreatus* is expanding into emerging areas, such as the fermentation of plant matrices for the development of plant-based ingredients. In this context, the microorganism can fully express its enzymatic potential directly on the food matrix. During fermentation, enzymes such as amylases, lipases, and proteases hydrolyze carbohydrates, lipids, and proteins into simpler and more digestible compounds, also contributing to the improvement of the product’s sensory characteristics (Dhull *et al.,* 2020).

Recent studies conducted at the University of Helsinki (Sankaran P., 2024) have investigated the effectiveness of *P. ostreatus* mediated SSF in the detoxification of lupin seeds. The study focuses particularly on the reduction of anti-nutritional fractions, such as quinolizidine alkaloids (QAs). The mechanism of alkaloid degradation, responsible for the bitterness and potential toxicity of the legume, is primarily attributed to the enzyme laccase. This ligninolytic enzyme catalyses oxidation processes capable of breaking the cyclic structures of alkaloids, transforming them into simpler, non-toxic intermediate metabolites. Consequently, the bio fermentation process reduces both the toxicity and the natural bitterness of the legumes.

Thanks to its ability to colonize solid substrates and produce a broad spectrum of extracellular enzymes, *P. ostreatus* is thus confirmed as an excellent microbial candidate for the application of SSF in the valorisation of plant matrices, contributing to the improvement of the nutritional value and functional properties of ingredients intended for the development of high-value plant-based foods.

2.4 Debittering treatment: Rational for a debittering free approach

Lupin seeds naturally contain approximately 70 alkaloids, many of which are toxic (Australia New Zealand Food Authority, 2001, pp. 1–21), particularly sparteine and lupanine (Jiménez-Martínez *et al.,* 2003), which must be removed before the consumption. They also contain other anti-nutritional factors, such as tannins and, notably, saponins, which are responsible for bitterness, can interfere with nutrient absorption, and may cause gastrointestinal irritation.

The primary and most widely used method for reducing alkaloids and saponins is debittering, which consists of three stages: soaking the seeds for 14–20 hours to increase their water content and facilitate alkaloid extraction, heat treatment in water at 90–95 °C for approximately 45 minutes, and prolonged washing in cold water for 5 days (20 L of water per kg of seeds, with daily water replacement). This aqueous approach is highly effective, capable of treating seeds with high alkaloid content (up to 4.2 g per 100 g of dry seed). However, it has some limitations: it requires large amounts of water (up to 63 kg per kg of seeds), long processing times (5–6 days), and results in significant solid losses (~0.27 kg per kg of seeds), reducing not only bitterness but also valuable nutrients, as the washing step is not selective (Carvajal-Larenas *et al.*, 2013).

For this reason, the present project adopts an alternative approach to traditional debittering, based on soaking the seeds in water for 24 hours (10 L of water per kg of seeds) followed by solid-state fermentation (SSF). This method exploits microbial activity to reduce alkaloids and saponins and represents a theoretically effective strategy for seeds with lower alkaloid content, specifically <1.1 g per 100 g of dry seed (Carvajal-Larenas *et al.*, 2013). In this way, the presence of bitter compounds can be reduced without the prolonged water washing and without the thermal treatment typical of conventional debittering, decreasing both treatment time and water consumption.

2.5 Technical pretreatments to improve SSF: PEF & HPU

In recent years, innovative non-thermal technologies have been explored as pre-treatments to enhance the fermentation of plant-based matrices, including lupin seeds. Among these, Pulsed Electric Fields (PEF) and High-Power Ultrasound (HPU) have gained attention for their ability to modify the structural properties of raw materials prior to microbial processing.

When applied before fermentation, these pre-treatments can improve substrate accessibility by altering cell integrity and promoting the release of intracellular compounds. This structural modification may facilitate microbial growth, enzyme activity, and mass transfer during fermentation, potentially leading to improved fermentation efficiency and enhanced biochemical transformations.

In the case of lupin seeds, the use of PEF and HPU as preliminary steps may support a more effective fermentation process, contributing to improved nutritional quality, reduced antinutritional factors, and better functional properties of the final product.

2.5.1 Pulsed Electric Field (PEF)

PEF treatment is a non-thermal food processing technology, as it generally causes only a minimal increase in temperature during application. PEF enhances the permeability of cell membranes by inducing the formation of pores, thereby facilitating the penetration of small molecules into the cytoplasm (Arshad *et al.,* 2021).

This phenomenon, known as electroporation, occurs through the application of short, high-voltage pulses, which can lead to either reversible or irreversible membrane permeabilization depending on the treatment conditions. The extent of pore formation is influenced by process parameters such as pulse duration (µs), electric field strength (kV/cm), number of pulses, and frequency (Hz) (Janositz *et al.,* 2011).

The application of PEF in fermentation involves the delivery of short, high-voltage direct current pulses to a biological sample placed between two electrodes within a treatment chamber. Typically, electric field strengths ranging from 0.1 to 5 kV/cm are applied with pulse durations between 50 and 1000 µs, although higher voltages (up to 40 kV) can also be used depending on the system (Galván-D'Alessandro and Carciochi, 2018).

From a biophysical perspective, the microbial cell membrane behaves as an electrical insulator because it is primarily composed of a phospholipid bilayer with hydrophobic fatty acid tails. This lipid core has very low electrical conductivity and a low dielectric constant compared to the surrounding aqueous media, which contain dissolved ions and are therefore good conductors. As a result, the membrane acts as a dielectric barrier separating two conductive regions, the intracellular and extracellular fluids, and can be described electrically as a capacitor.

When a cell suspension is exposed to an external electric field, ions present in the conductive media migrate along the field lines and accumulate at both sides of the membrane. This charge accumulation leads to the development of a transmembrane potential. As the intensity of the applied electric field increases, the induced transmembrane potential also rises. If this potential exceeds a critical threshold (typically 0.2–1 V), the structural stability of the lipid bilayer is disrupted, causing a temporary loss of membrane semi permeability. This phenomenon, known as electroporation results in the formation of pores that can be either reversible or irreversible depending on the strength and duration of the applied electric field.

Electroporation is particularly useful in food processing because it increases cell membrane permeability. This controlled permeabilization facilitates the extraction of intracellular components such as polyphenols, pigments, sugars, and other bioactive compounds, thereby improving yield and efficiency in processes like juice production and fermentation, where the release of substrates enhances microbial activity. At the same time, when applied at higher intensities, electroporation can irreversibly damage microbial cells, leading to their inactivation. For this reason, PEF is also considered a promising non-thermal alternative to conventional pasteurization, ensuring microbial safety while better preserving nutritional and sensory quality (Arshad *et al.*, 2021). The application of PEF can be observed in the figure 1.2.

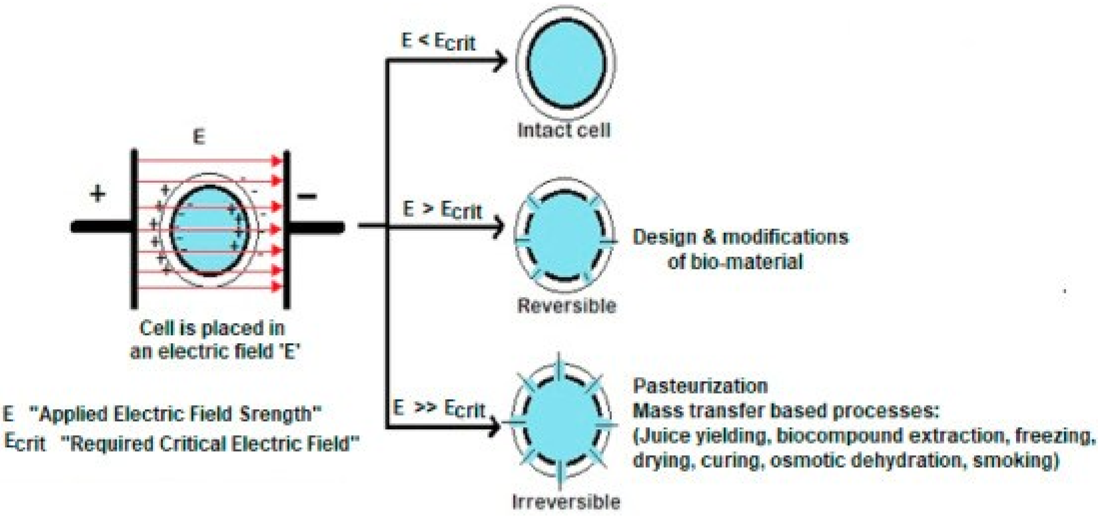


Fig 1.2: *Arshad et al., 2021*
Process of cell membrane permeabilization and functional application.

In this project, PEF is applied as a versatile technology to enhance SSF processes and boost industrial production of PBMAs.

2.5.2 High Power Ultrasounds: HPU

In addition to use the Pulsed Electric Fields, the other technology applicated in this project is the High-Power Ultrasounds (HPU). It consists in the phenomena of cavitation that refers to the formation, growth and collapse of vapour-filled cavities in a liquid caused by the propagation of high-intensity ultrasonic waves.

In this case, the application of ultrasounds produced by the ultrasonic transducers does not affect directly the lupine seeds but affects the surrounding liquid, usually water, present in the chamber. After that, the cavitation and the rupture of the bubbles finally affect the cellular membrane of the seeds.

When the ultrasonic waves, produced by the transducers, pass through the liquid medium, they induce a longitudinal displacement of particles, whereas the source of the sound wave acts as a piston, resulting in a succession of compression and rarefaction phases in the medium. If the rarefaction phase is sufficiently intense, the distance between neighbouring liquid molecules can approach or exceed a critical point, creating voids in the liquid known as cavitation bubbles. These initial bubbles can expand during rarefaction, when the local pressure drops, and shrink during compression cycles, when the local pressure rises, until they reach a critical size and collapse violently, releasing high local pressures and temperatures *(*Galván-D'Alessandro and Carciochi, 2018). Depending on the ultrasound frequency, cavitation in the liquid can be transient or stable. Low frequencies (20–100 kHz) cause transient cavitation, with bubbles rapidly growing and collapsing violently, generating intense local pressures and temperatures. Higher frequencies (>200 kHz) produce stable cavitation, where bubbles oscillate over many cycles, resulting in milder mechanical effects.

The collapsing bubbles can generate extremely high pressures and temperatures, suggested to be respectively up to 1700 atm and up to 5000 K, as represented in Figure 1.3.

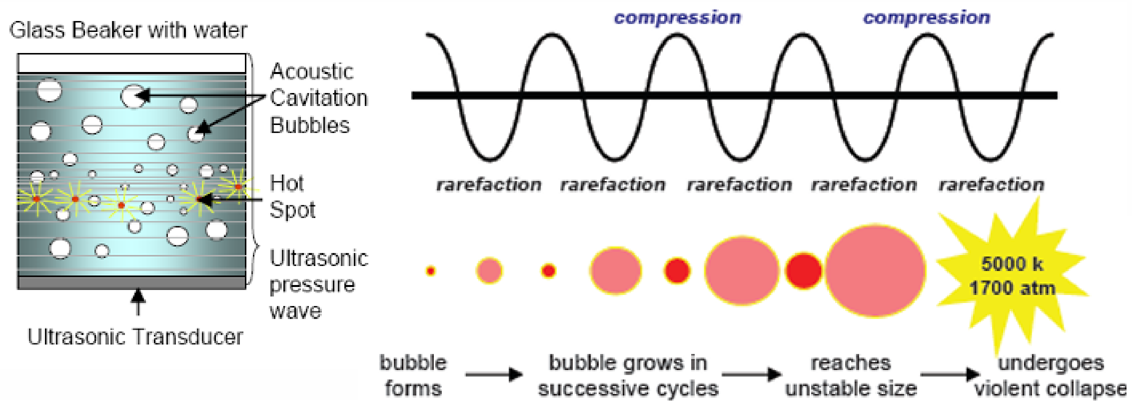


Fig 1.3: *Lorenzetti, 2024, slides of Processi chimici innovativi, University of Padua*
Process of bubble cavitation: formation and collapse

Ultrasound used as a substrate pre-treatment before solid-state fermentation can enhance the process by inducing cavitation effects that disrupt cell walls, increase surface porosity, and improve the release of intracellular compounds. This structural modification enhances nutrient accessibility and mass transfer, facilitating microbial colonization and potentially accelerating fermentation kinetics and metabolite production.

Physically, the cavitation process produces shockwaves and microjets, subjecting the biomass to intense hydromechanical shear forces that further break down the complex matrix. According to *Nemes et al.2025,* the use of HPU as pretreatment of SSF significantly overcomes the structural resistance of the substrate. The mechanical action of acoustic cavitation leads to the disruption of cell walls, effectively releasing bioactive compounds that are typically trapped within the matrix. It leads to an enhanced phenolic release, to an improved sugar yield and to an increased process efficiency: by making the substrate more porous and accessible, ultrasounds accelerate the metabolic activity of the fungi, leading to a more efficient bioconversion of byproducts into high value functional ingredients.

In this project, PEF and HPU are applied as pre-treatments prior to the subsequent SSF of lupine seeds with *P. ostreatus*. The fermentation outcomes are monitored over time and compared with a control SSF conducted without pre-treatment (CTRL), to evaluate whether these alternative steps effectively improve process efficiency.

Chapter 3

Materials and Methodology

This chapter describes the materials, methods, and experimental approaches used in this project. It provides a detailed overview of the raw materials and of the procedures applied for the SSF of lupin seeds with *P. ostreatus* for each pretreatment employed (CTRL, PEF, and HPU). Furthermore, this chapter outlines the analytical methods and the statistical approaches applied throughout the study. Understanding these protocols is essential for following the experimental design and ensuring reproducibility of the fermentation processes for each pretreatment.

3.1 Raw materials

For this study, lupin seeds (*Lupinus albus* cv. Estoril) were provided by Fertiprado (Badajoz, Spain), and the mycelium of *P.ostreatus* M2181 was obtained from MYCELIA (Belgium). Fermentation was conducted in Microbox Sac O_2 units (Deinze, Belgium), which were equipped with a filtration system that permits air exchange while preventing contamination, ensuring controlled and sterile conditions throughout the process.

3.2 Reagents

The following products were used for the analytical determinations carried out in this study: The following products were used for the analytical determinations: sulfuric acid 72 % (H_2SO_4, Fisher Scientific Spain), sodium hydroxide 10 M and 0.5 M (NaOH, TCI Chemicals), acetylacetone reagent prepared from 1 mL of acetylacetone ($C_5H_8O_2$, Scharlab, AC02200250) and 50 mL of 0.5 M sodium carbonate (Na_2CO_3, Scharlab, SO01161000), ethanol (C_2H_6O, Scharlab), Ehrlich reagent prepared from 2.67 g of p-dimethylaminobenzaldehyde ($C_9H_{11}NO$, Merck, 156477) and an ethanol:HCl 1:1 solution (50 mL ethanol + 50 mL hydrochloric acid, HCl) (Scharlab, AC07411000), glucosamine hydrochloride ($C_6H_{13}NO_5 \cdot HCl$, Merck, PHR1199, CAS 66-84-2), D-(+)-glucosamine hydrochloride 98.0+ % ($C_6H_{13}NO_5 \cdot HCl$, Fisher Scientific Spain, TCI America™, G004425G, CAS 66-84-2), Folin–Ciocalteu reagent (mixture of sodium tungstate Na_2WO_4 and sodium molybdate Na_2MoO_4 in phosphoric acid) (Merck), acidic methanol (mixture of methanol CH_4O and 0.2 M HCl) (Scharlab), gallic acid ($C_7H_6O_5$, Merck), anhydrous sodium carbonate 20 % w/v (Na_2CO_3, Scharlab, SO01161000), distilled water, 0.3 M anhydrous sodium acetate buffer pH 3.6 prepared from 0.155 g sodium acetate ($C_2H_3NaO_2$, Scharlab) and glacial acetic acid ($C_2H_4O_2$, Scharlab), 20 mM ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, Merck, 236489), 10 mM TPTZ in 40 mM HCl ($C_{18}H_{12}N_6$, Merck, 93285), Trolox ($C_{14}H_{18}O_4$, Merck), sodium acetate ($C_2H_3NaO_2$, Scharlab), vanillin ($C_8H_8O_3$, Sigma-Aldrich, CAS 121-33-5), sulfuric acid (H_2SO_4, Scharlab, AC20691000), oleanolic acid ($C_{30}H_{48}O_3$, Merck, 42515), methanol (CH_4O, Scharlab), dichloromethane (CH_2Cl_2, Scharlab), sodium sulfate (Na_2SO_4, Scharlab), anhydrous sodium sulfate (Na_2SO_4, Scharlab, SO06641000), ethyl tetrabromo-phenolphthalein ($C_{20}H_{10}Br_4O_4$, Merck, 86778), p-toluenesulfonic acid monohydrate ($C_7H_8SO_3H \cdot H_2O$, Merck, 402885), disodium phosphate (Na_2HPO_4, Scharlab, SO03370500), monosodium phosphate (NaH_2PO_4, Scharlab, SO03301000), BCA protein assay kit (Thermo Scientific, BCA1), sunflower oil (Mercadona), and sodium dodecyl sulfate (SDS, $C_{12}H_{25}SO_4Na$, Scharlab).

3.3 Sample preparation, Fungi inoculation and SSF

Considering the experimental part, the process of Solid-State Fermentation is represented in Figure 3.1.

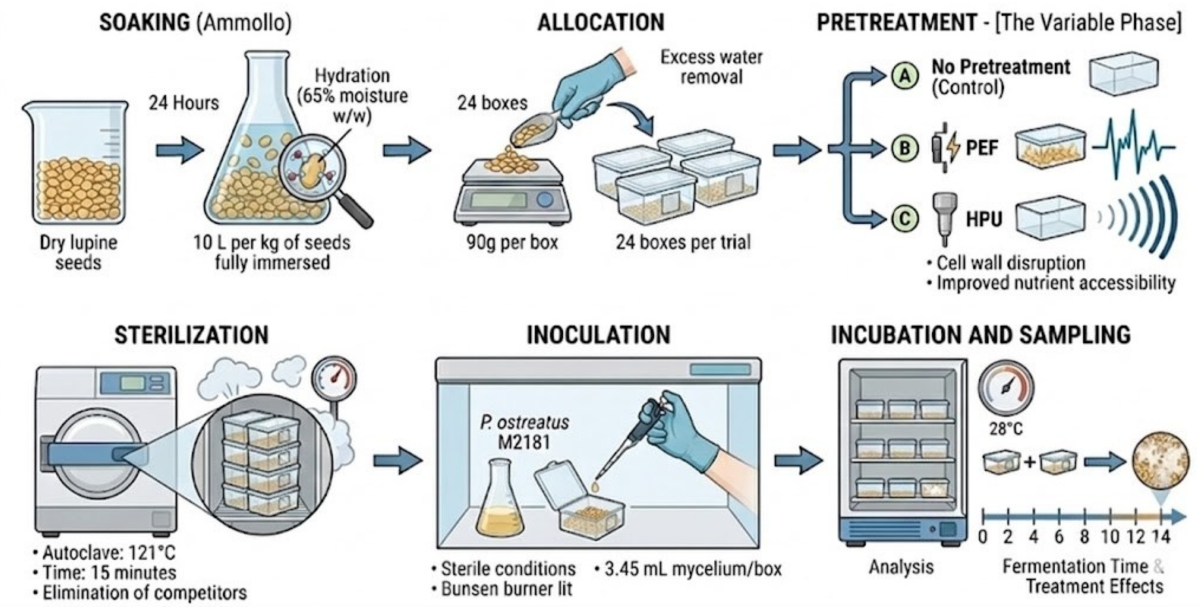


Fig 3.1: *Schematic representation of the preparation steps for the SSF*
Image generated using a generative AI system (Google Gemini, 2026)

As described in the previous chapter, this project adopted an alternative approach to traditional debittering, based on soaking the seeds in water for 24 hours (10 L per kg of seeds). This step was crucial, as it brought the lupine seeds to approximately 65% moisture content (w/w), ensuring uniform hydration, improved nutrient accessibility, and optimal microbial growth. After soaking, the excess water was removed, and 90 g of lupine seeds were transferred into each microbox. For each pretreatment, a total of 24 microboxes are prepared, corresponding to 3 independent samples per day of fermentation across 8 fermentation days (0, 2, 4, 6, 8, 10, 12, 14). After this allocation, each microbox was subjected to its respective pretreatment (no pretreatment, PEF, or HPU).

The last step that occurred before the inoculation of the *P. ostreatus* was the sterilization step, where the samples were sterilized by autoclaving at 121 °C for 15 minutes.

Following sterilization, the seeds were allowed to cool to room temperature before being inoculated with the fungus *P. ostreatus*. The study employs the *P. ostreatus* M2181, a mycelium widely used in solid-state fermentation research, obtained from a commercial culture and stored at 4 °C until use.

All manipulations were carried out in a laminar flow hood, with a lit Bunsen burner and using previously sterilized materials to prevent any contamination. Each microbox received 3.45 mL of mycelium, applied drop by drop evenly over the seeds.

After inoculation, each microbox was placed in an incubator at 28 °C and removed at the predetermined time points for each box, allowing the evaluation of the effects of both fermentation duration and the pretreatment applied.

The effects of the different days of fermentation for the PEF samples are represented in Figure 3.2.

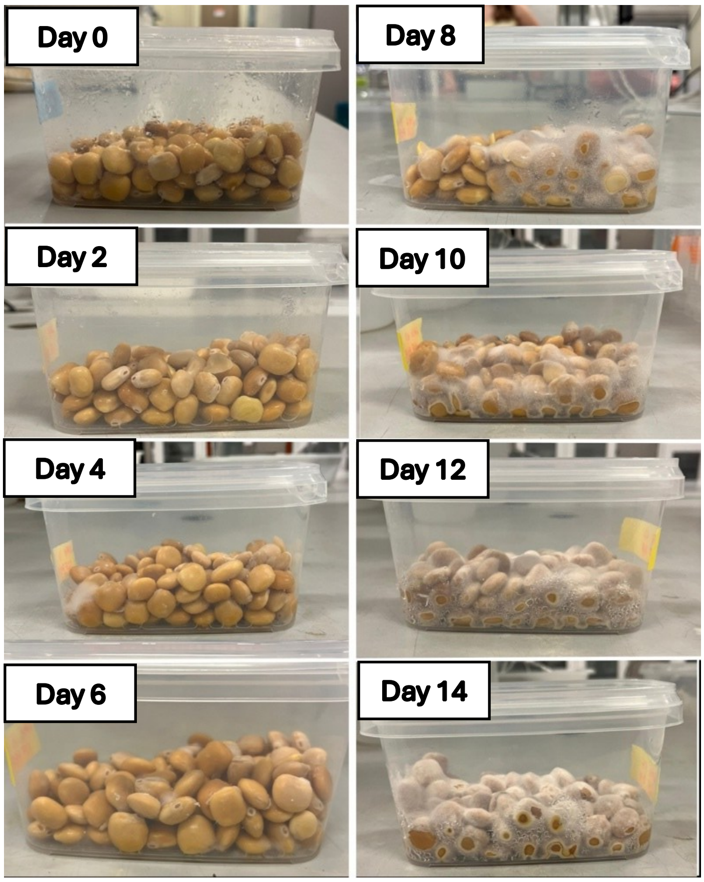


Fig 3.2: *Evolution of SSF with P.ostreatus in lupine seeds pretreated with PEF*

3.4 Technical pretreatments (PEF and HPU)

The main concept of this project is to evaluate the results of the SSF over the days and over the different pretreatments applicated to the samples. For this reason, is essential to improve and to ensure the reproducibility for each kind of pretreatment (CTRL, PEF and HPU).

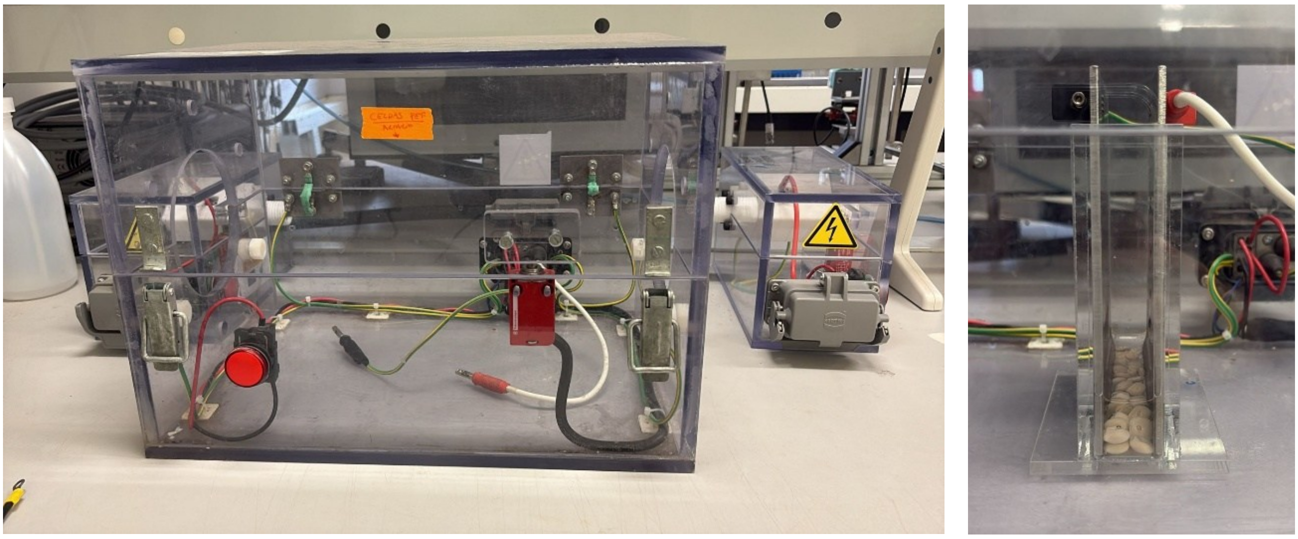
The CTRL condition simply involves skipping the technological pre-treatment step, whereas different specific procedures are applied for the other technological treatments.

3.4.1 Technological setup of PEF

After the soaking and after the allocation of seeds in each microbox, the lupine seeds subjected to Pulsed Electric Field (PEF) pre-treatment were exposed to an electric field strength of approximately ±6 kV/cm. A total of 300 pulses is applied at a frequency of 10 Hz, with a pulse width of 24 µs and a power of 3000 W.

For each treatment cycle, 15 g of seeds and 30 mL of distillate water were placed in the treatment chamber prior to pulse application. The treatment was carried out using an EPULSUS-BM1A-12 system (EnergyPulseSystems, Lisbon, Portugal).

The experimental setup used for the PEF pre-treatment of lupine seeds is shown in Figure 3.3.



(a) *(b)*

Fig 3.3: *(a) Structural chamber for PEF application. (b) Electrodes for PEF*

3.4.2 Technological setup of HPU

After the soaking and after the allocation of seeds in each microbox, the lupine seeds subjected to HPU pre-treatment were processed in batches. For each ultrasonic step, 80 g of seeds were placed into the reaction vessel containing 400 mL of distilled water. The ultrasonic probe (sonotrode) was submerged into the liquid to a depth of approximately 3 cm.

The seeds were then exposed to a continuous ultrasonic field (100% duty cycle) at maximum amplitude (100%), with a constant power output of 185 W for a duration of 10 minutes.

The process temperature was strictly controlled at a setpoint of 50 °C. Whenever this threshold was exceeded, cooling water at 4 °C was automatically circulated through the container's cooling coil to dissipate the heat generated by acoustic cavitation and maintain isothermal conditions. The ultrasonic pre-treatment was performed using a Hielscher UP400St High-

Power Ultrasound (HPU) processor, a state-of-the-art device with a maximum power capacity of 400 W. This system was specifically used to facilitate the subsequent fermentation process by partially breaking down the lupine cellular matrices.

The experimental setup used for the HPU pre-treatment of lupine seeds is shown in Figure 3.4.

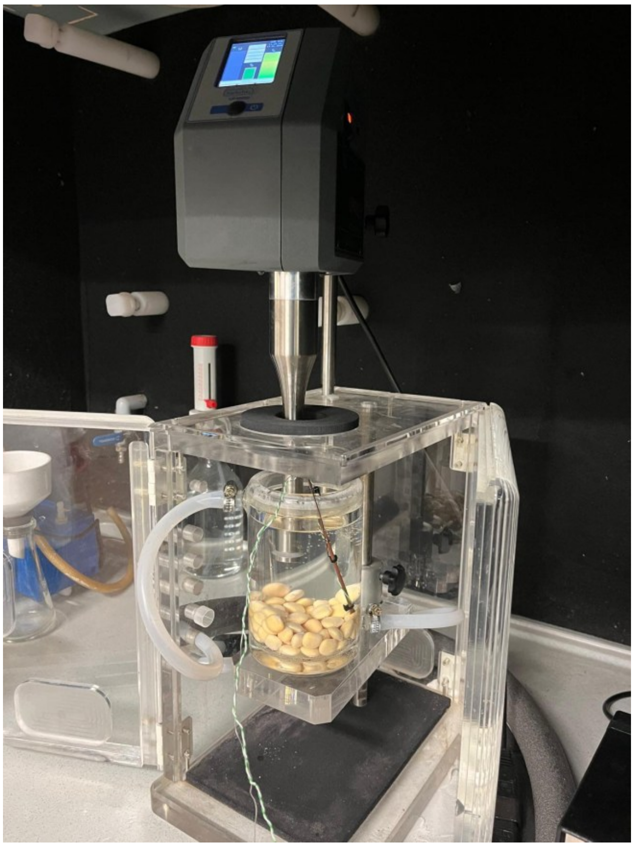


Fig 3.4: Setup and application of HPU on lupine seeds

3.5 Analytical determinations

After the fermentation process, all samples were frozen at –25 °C and subsequently lyophilized for three days. The freeze-drying was carried out using a lyophilizer (LyoMicron, Coolvacuum) operating at a vacuum pressure of 0.065 mbar, with the condenser maintained at approximately –85 °C. After lyophilization, each sample was homogenized by grinding with a mill (KG210, DeLonghi) until a fine powder was obtained. This powdered material was then used for the various analytical determinations.

To evaluate the effectiveness of the fermentation process and the impact of the different pre-treatments, several key parameters were monitored throughout the fermentation period. First, it was important to quantify the total amount of glucosamine produced, that is directly connected to the growth of the fungi *P. ostreatus* during the time. In addition, the evolution of both soluble and total protein content in the lupine seeds was analysed during fermentation in order to assess potential improvements in these important nutritional parameters.

Furthermore, to better evaluate the efficiency of the fermentation process, the trends of the main antinutritional compounds naturally present in lupine seeds were also investigated, including alkaloids, saponins, and polyphenols. Antioxidant activity and fat content were also determined to provide a more comprehensive evaluation of the biochemical changes occurring during fermentation.

3.5.1 Glucosamine determination

Chitin is a key structural polysaccharide present in the cell walls of filamentous fungi such as *P. ostreatus*. It represents an ideal marker for the quantification of fungal biomass because it is almost exclusively found in fungi, providing high specificity. Chemically, chitin is a polymer of N-acetylglucosamine that, upon acid hydrolysis, releases glucosamine. Following the method applicated in Sànchez-Garcia *et al.* (2022), the amount of glucosamine released from chitin can be used to estimate fungal biomass. In this study, the released glucosamine was quantified using a spectrophotometric method.

For the analysis, 100 mg of sample were weighed for each replicate, and 2.4 mL of 72% H_2SO_4 were added. The strong acid promotes the hydrolysis of chitin in the fungal cell wall, leading to the release of glucosamine. The samples were then incubated at 25 °C for 24 h to allow the hydrolysis to proceed. After incubation, the samples were diluted with 55 mL of distilled water and subsequently autoclaved at 121 °C for 15 min. This high-temperature treatment ensures the completion of the acid hydrolysis, allowing the complete release of glucosamine and enabling a more accurate quantification of fungal biomass.

Following hydrolysis, the pH of the samples was neutralized to pH 7 using 10 M NaOH and 0.5 M NaOH, as the colorimetric assay requires neutral conditions. The total volume of each sample was measured. After pH adjustment and the volume quantification, 1 mL of each sample solution was transferred to a new tube and mixed with 1 mL of acetylacetone reagent (prepared with 1 mL acetylacetone in 50 mL of 0.5 M sodium carbonate solution). The mixtures were then incubated at 100 °C for 20 min.

After incubation, 6 mL of ethanol and 1 mL of Ehrlich reagent (2.67 g of p-dimethylaminobenzaldehyde dissolved in an ethanol: HCl solution, 1:1 v/v) were added to each sample. The solutions were thoroughly mixed using a vortex mixer and incubated at 65 °C for 10 min to allow colour development. After cooling, the absorbance was measured at 530 nm using a spectrophotometer. The absorbance values were used to determine the glucosamine concentration, which served as an indirect estimate of fungal biomass.

For the quantification of glucosamine content, a calibration curve (Figure 3.5) was prepared following the procedure described above,but replacing the hydrolysed sample with known concentrations of N-acetyl-D-glucosamine ranging from 0 to 0.05 mg/mL.

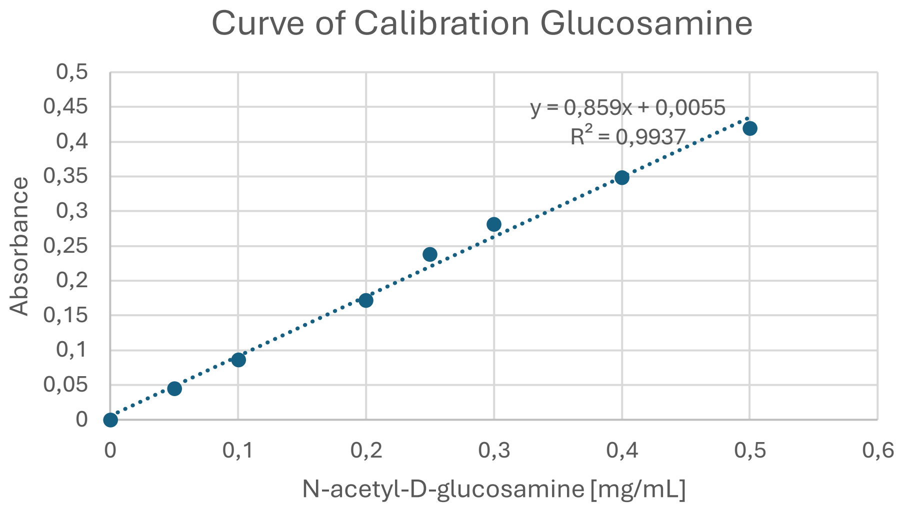


Fig 3.5: *Curve of calibration for glucosamine analysis*

The results were expressed as milligrams of glucosamine per gram of dry sample.

3.5.2 *Total proteins*

The total protein content of the samples was determined using the Dumas method (AOAC 992.23) with a LECO CN628 analyzer. Nitrogen content (%N) was measured, and crude protein content (%CP) was subsequently calculated using the standard conversion factor of 6.25.

3.5.3 *Alkaloids*

For the determination of alkaloids content, the method utilized in Garzera *et al.* (2010) was employed. In this method the determination of alkaloids is based on an acid–base titration, a volumetric method in which p-toluenesulfonic acid acts as the titrating reagent and reacts with the alkaloids present in the sample. As the acid is added, the alkaloids are progressively protonated, resulting in a change in the pH of the solution. The endpoint of the titration is detected using a colorimetric indicator (ethylbromo-phenolphthaleinate), which undergoes a color change when all the alkaloids have been protonated, indicating that the titration is complete.

Considering the extraction phase, for each replicate, 0.25 g of sample was extracted with 7.5 mL of 0.5 N HCl. The mixture was agitated magnetically for 30 minutes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected, and the pellet was resuspended in an additional 7.5 mL of 0.5 N HCl, agitated for another 30 minutes, and centrifuged again under the same conditions. The second supernatant was combined with the first. After this, the ph of each solution was adjusted at 10 with NaOH 4N. Successively for each solution 12.5 mL of dichloromethane were added. After a centrifugate at 7200g for 15 minutes the organic phase was recovered. The addition of dichloromethane to the aqueous phase and the centrifuge were repeated and the new organic phase was combined with the first. A filter with anhydrous sodium sulphate and glass wool was created and used to absorb any remaining water present in the organic solution, as showed in Figure 3.7. The last step of the extraction was the evaporation of dichloromethane, obtained leaving the samples in the oven at 40 °C for one night.

Fig 3.7: Filtration of organic phase for alkaloids extraction

After the extraction, the titration was carried out by dissolving the organic sample in 5 mL of methanol. Three drops of the indicator (ethylbromo-phenolphthaleinate) were added and thoroughly mixed with the solution. The main part of the analysis consisted of adding the titration solution (194 mg of p-toluenesulfonic acid in 100 mL of methanol) drop by drop until a colour change from blue to yellow was observed. When the yellow colour remained stable, the titration was considered complete.

The volume of titrant used was considered proportional to the total alkaloid content in the sample, as one equivalent of acid reacts with one equivalent of alkaloid. From this, the total alkaloid content can be calculated and expressed as lupanine equivalents, using lupanine as the reference compound.

The total alkaloid content was expressed as g of lupanine per 100 g of sample, assuming that each lupanine molecule contains a single basic equivalent capable of reacting with the acid.

3.5.4 Saponins

For the determination of saponins content the method utilized by Navarro de Hierro *et al.* (2018) was used. This method uses 8% (v/v) vanillin as a chromogenic reagent, which reacts with the sapogenin portion of the saponins to produce a coloured complex.

For each replicate, 0.33g of sample was extracted and added to 10 mL of distillate water. Subsequently, 30 minutes of magnetic agitation and 10 minutes of centrifugation at 6000 rpm were applicated. After that, the supernatant with a dilution 1:30 was extracted.

For the analysis, 100 µL of sample was mixed with 100 µL of 8% (v/v) vanillin and 1 mL of H_2SO_4. The solution was then placed in an oven at 60 °C for 10 minutes to allow the reaction between vanillin and the sapogenin portion of the saponins. After cooling, quartz cuvettes were used to withstand the acids and organic solvents, and the absorbance was measured at 540 nm using a spectrophotometer.

For the quantification of saponin content, a calibration curve (Figure 3.8) was prepared using different concentrations of oleanolic acid in ethanol as the standard reference (ranging from 0 to 1.25mg/mL)

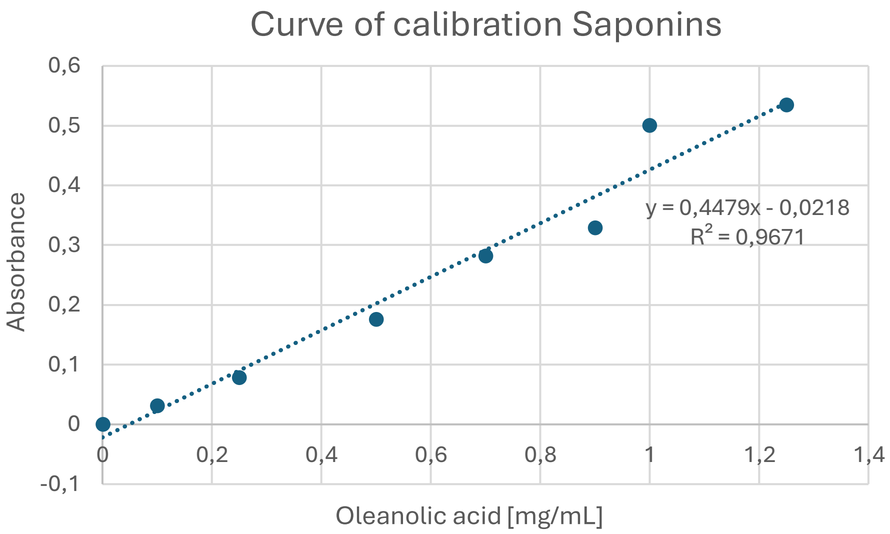


Fig 3.8: *Curve of calibration for Saponins analysis*

The results were expressed as grams of oleanolic acid per gram of dry sample.

3.5.5 Polyphenols and Antioxidant activity

Polyphenols are among the key antioxidant components of the sample, contributing to its overall ability to neutralize free radicals. In this study, both the total polyphenol content and the overall antioxidant capacity were evaluated to assess the functional properties of the lupine seeds during fermentation.

For the quantification of total polyphenols and antioxidant activity, the methodology developed by Navarro-Vozmediano *et al.* (2024) was employed.

For the extraction step, the procedure was the same for both analyses. For each replicate, 0.5 g of sample was mixed with 5 mL of 0.2 M acidified methanol (prepared with 500 mL of methanol and 8.2 mL of 37% (w/w) HCl). The mixture was first homogenized using a vortex and then placed on a magnetic stirrer (Rotabit, PSelecta) for 15 minutes at 125 rpm.

Subsequently, the samples were placed in an ultrasonic bath (Ultrasonic Cleaner USC_T, VWR) for 15 minutes in the dark. After sonication, the samples were centrifuged at 10,000 rpm and

4°C for 10 minutes. The supernatant was then recovered and filtered through a 0.45 µm filter (Filter-Lab, Barcelona, Spain) to obtain the final extract.

Considering the polyphenols analysis, the Folin-Ciocalteau method was applied. Using Eppendorf tubes of 2 mL, following reactants were mixed for each sample: 50 µL of Folin–Ciocalteu reagent, 10 µL of calibration standards or samples, 100 µL of 20% (w/v) anhydrous Na_2CO_3 solution, and 840 µL of distilled water were mixed. The mixtures were then vortexed and incubated for 20 minutes at room temperature in the dark. After that, 200 µL of each solution were recovered, and the absorbance was measured at 700 nm using a spectrophotometer.

The calibration standards (Figure 3.9) were prepared using different concentrations of gallic acid (ranging from 0 to 750 mg/L) dissolved in acidified methanol as the solvent.

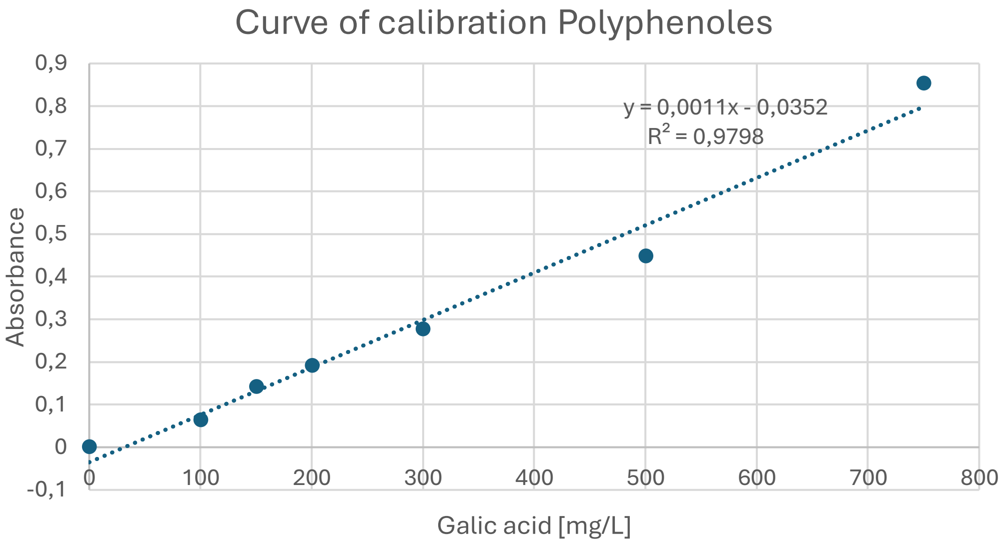


Fig 3.9: *Curve of calibration for Polyphenoles analysis*

The results were expressed as milligrams of equivalent gallic acid per gram of dry sample.

Considering the Antioxidant Activity, the method base on the Ferric Reducing Antioxidant Power (FRAP) was used. This reactant measures the reduction of a colourless complex formed by a chromogenic reagent, in this case TPTZ (2,4,6-tripyridyl-s-triazine), and Fe^{3+} to a ferrous complex (Fe^{2+}) with a blue-green coloration in the presence of antioxidant compounds under acidic conditions.

The FRAP reagent was prepared by mixing three different solutions in fixed proportions: 0.3M anhydrous sodium acetate buffer, 20 mM $FeCl_3 \cdot 6H_2O$, and 10 mM TPTZ. For each sample, 10 µL of extract were mixed with 180 µL of FRAP reagent. The mixtures were then incubated in an oven at 37 °C for 30 minutes to allow the reaction to occur. After incubation and cooling, the absorbance was measured at 595 nm using a spectrophotometer.

The calibration standards (Figure 3.10) were prepared using different concentrations of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) ranging from 0 to 600 µM, dissolved in acidified methanol as the solvent.

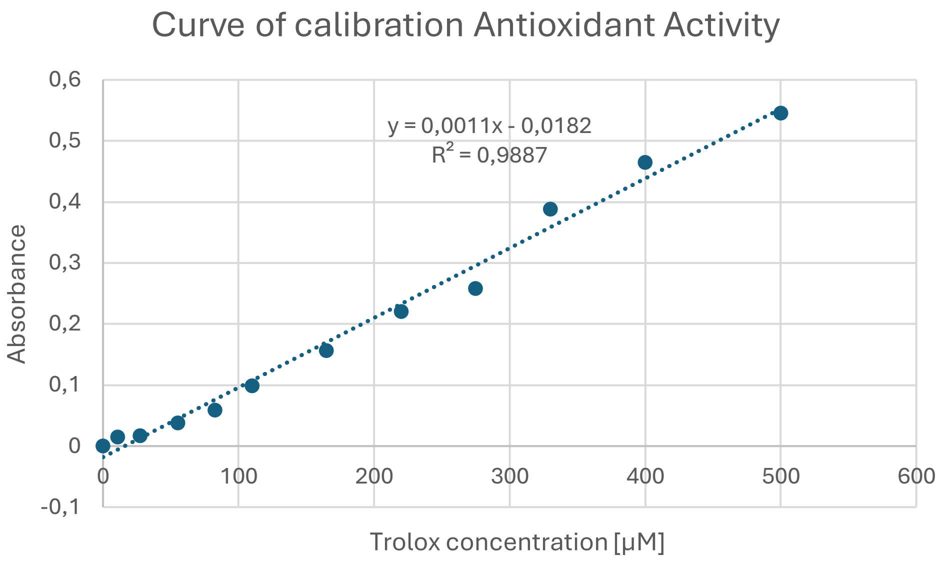


Fig 3.10: *Curve of calibration for Antioxidant Activity analysis*

The results were expressed as milligrams of equivalent trolox per gram of dry sample.

3.5.6 Fat content

The fat content was determined using the Soxhlet extraction method, following the AOAC procedure (991.36) (AOAC, 1996), and employing a Soxtec™ 2055 extraction unit (FOSS). Results were expressed as grams of fat per 100 grams of sample.

For each replicate, 1 g of sample was dried in an oven at 100 °C overnight to achieve complete dryness. The dried samples were then placed in cylindrical extraction tubes and immersed in grey tubes with petroleum ether, which served as the solvent. The Soxtec™ 2055 unit operated through four sequential steps at 115 °C: the first step (140 min) for fat extraction into the solvent, the second step (90 min) and third step (11 min) for solvent evaporation, and a final step (20 min) to cool the tubes. After extraction, the tubes containing only the extracted fat were weighed. They were then cleaned, and the empty tubes were weighed again, allowing the fat content of the samples to be calculated gravimetrically by the difference in weight. The machine used is shown in Figure 3.11.

Fig 3.11: *Soxtec™ 2055 used for fat content extraction*

3.6 Techno-functional Properties

The techno-functional properties of the samples were also evaluated, including gelation capacity, water and oil absorption capacity, foaming capacity, and emulsifying capacity. These properties are important indicators of the technological behaviour of plant-based ingredients and determine their potential applications in food formulations. In particular, they influence texture, moisture retention, fat binding, and the stability of air–water or oil–water systems. Therefore, their evaluation is essential to assess the suitability of fermented lupine as an ingredient for the development of PBMAs.

3.6.1 Soluble proteins

For the analysis of soluble proteins, the method based on bicinchoninic acid (BCA) described by Shen *et al.* (2023) was used. In this method soluble proteins reduce Cu^{2+} ions to Cu^{1+}, which then reacts with bicinchoninic acid to form a stable, purple-coloured complex. The intensity of the colour increases proportionally over a wide range of protein concentrations, allowing accurate quantification. For each replicate, 1 g of sample was extracted and diluted in 10 mL of distilled water. The mixture was mechanically agitated for 30 minutes and then centrifuged at 10,000 rpm for 10 minutes to recover the supernatant. From this, 1 mL of the solution was transferred to 15 mL conical centrifuge tubes (Falcon®), and 3 mL of distilled water were added to achieve a 1:40 dilution.

Subsequently, 2 mL of BCA reagent (prepared as a 1:50 dilution of $CuSO_4$ and bicinchoninic acid) was added to 100 µL of each diluted sample. The mixtures were incubated in an oven at 37 °C for 15 minutes. After cooling, 200 µL of each solution were recovered, and the absorbance was measured at 562 nm using a spectrophotometer.

In this case the curve of calibration (Figure 3.6) was obtained using bovine serum albumin (BSA) as standard protein (0–1000 µg/mL).

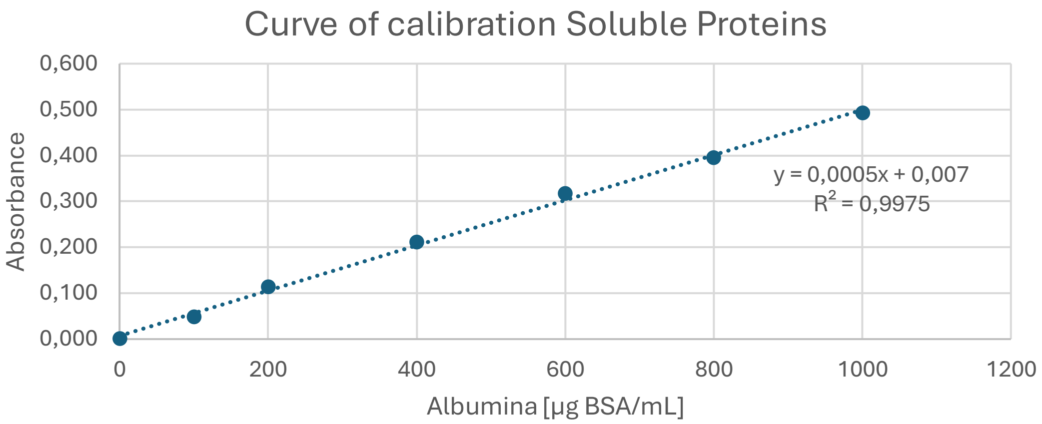


Fig 3.6: *Curve of calibration for soluble proteins analysis*

The results were expressed as grams of glucosamine per gram of dry sample.

3.6.2 Gelation Capacity

Gelation capacity represents a key parameter for achieving a texture similar to that of meat and is therefore essential for the development of effective plant-based meat alternatives (PBMAs). In this study, gelation capacity was determined according to the method described by Locali-Pereira *et al.* (2022), with minor modifications.

For each sample, ten different concentrations ranging from 2% to 20% (w/v) were prepared by dispersing the different amount of sample in 5 mL of 50 mM phosphate buffer (pH 7). Each dispersion was vortexed twice for 30 s, with a resting period of 5 min between mixing steps to ensure proper hydration of the sample. The dispersions were then immersed in a boiling water bath at 100 °C for 1 h, as shown in Figure 3.12.

(a) *(b)*

Fig 3.12: *Gelation capacity procedure*
(a) Boiling water bath (b) Prepared dispersions

Subsequently, the tubes were stored at 4 °C for 24 h. The following day, each tube was inverted to evaluate gel formation at the different concentrations for each sample. The minimum sample concentration capable of forming a gel that did not flow upon tube inversion was considered the least gelation concentration (LGC).

3.6.3 Water absorption Capacity and Oil absorption Capacity

Water absorption capacity (WAC) and oil absorption capacity (OAC) were determined according to the method described by Navarro-Vozmediano et al. (2024). For each sample, 0.5 g of extract was weighed and placed in a 15 mL Falcon tube. The weight of the extract (PE) and the combined weight of the extract and tube (PO) were recorded. Subsequently, 5 mL of either distilled water or oil were added to each tube, and the mixture was vortexed for 1 min. The samples were then allowed to stand at room temperature for 30 min and subsequently centrifuged at 1600 g for 25 min.

Each sample was then inverted over filter paper and allowed to drain for 20–30 seconds to remove any unbound water or oil. The tubes were then weighed again (PF), and the absorption capacity was calculated using Equation 3.1.

$$Water\ or\ oil\ capacity = \frac{PF - PO}{PE} \tag{3.1}$$

The calculated values represent the amount of water or oil absorbed per gram of sample.

3.6.4 Foaming and Emulsifying Stability

The methodology used for both assays was based on the method described by Navarro-Vozmediano *et al.* (2024).

Considering the Foaming Capacity, for each replicate 0.3g of sample were extracted and mixed with 15 mL of distillate water in a 50 mL Falcon tube. The weight of the sample and the tube were recorded, and the initial height of the liquid in the tube was measured. The dispersion was then homogenized for 1 min at 12,000 rpm using an Ultra-Turrax homogenizer (rotor S25N–18G ST, Ultra-Turrax T25, IKA), as shown in Figure 3.13.

Fig 3.13: *Ultra-Turrax homogenizer, for foaming homogenization*

After homogenization, the total height of the foam and the radius of the cylindrical Falcon tube were measured in order to calculate the foam volume using the following equation:

$$V_{foam} = \pi \cdot r^2 \cdot h \tag{3.2}$$

Foaming capacity was calculated as shown in Equation 3.2.

$$F_o = \frac{V_{foam}}{V_{sample}} \ o \ F_o = \frac{V_{foam}}{g_{protein}} \tag{3.3}$$

Foaming stability index (FSI) was calculated as the ratio between the foam volume at a given time and the initial foam volume, expressed as a percentage:

$$FSI = \left(\frac{V_{foam}(t)}{V_{starting\ foam}}\right) \cdot 100 \tag{3.4}$$

Considering the emulsifying capacity, this parameter represents an important property for PBMAs, as it reflects the ability of proteins to stabilize oil-in-water emulsions, which are essential for achieving proper fat distribution and texture in the final product.

To evaluate this property, 0.5 g of each sample was weighed and mixed with 50 mL of distilled water. From this dispersion, 6 mL were transferred to a 50 mL Falcon tube and mixed with 2 mL of oil. The mixture was then homogenized for 1 min at 13,500 rpm using an Ultra-Turrax T25 homogenizer (IKA).

Subsequently, 20 µL of the emulsion were taken from the bottom of the tube and mixed with 1.5 mL of 0.1% (w/v) sodium dodecyl sulfate (SDS) solution and homogenized. The absorbance was measured at 500 nm using a microplate reader (EZ Read 2000, Biochrom). After 10 min, the absorbance was measured again.

Using these two measurements, it was possible to calculate the Emulsifying Activity Index (EAI), which reflects the ability of the sample to create an emulsion, and the Emulsion Stability Index (ESI), which represents the ability of the emulsion to remain stable over time.

$$EAI = \frac{2 \cdot T \cdot F}{\varphi \cdot C} \tag{3.5}$$

$$ESI = \frac{EAI_{t=10min}}{EAI_{t0}} \tag{3.6}$$

Where:

- Optical path length of the cuvette L=0.007 m
- Turbidity $T = 2.303 \cdot A_{500} \cdot L$
- Dilution Factor F= 1520 µL/20 µL
- Oil volume fraction $\varphi = 0.25$
- Protein concentration C=1 mg/mL

3.7 Statistical approach

All experimental results are presented as the mean ± standard deviation (SD) of three independent replicates. Statistical analyses were performed using Statgraphics Centurion XIX. The effects of pretreatment type and fermentation time, as well as their interaction, were evaluated using a two-way ANOVA ($p < 0.05$). When significant effects were observed, one-way ANOVA was applied to further investigate differences either among fermentation times within the same pretreatment or among pretreatments within the same fermentation time. Homogeneous groups were identified using Tukey's Honestly Significant Difference (Tukey HSD) test ($p < 0.05$).

In the figures, homogeneous groups are indicated by letters: lowercase letters denote statistically significant differences among fermentation times within the same pretreatment, whereas uppercase letters indicate statistically significant differences among pretreatments within the same fermentation time.

Chapter 4
Experimental Results

This chapter presents the results obtained in this study. First, the growth of *P. ostreatus* was evaluated over time under different pre-treatment conditions. Subsequently, the analyses provided relevant information on the content of quinolizidine alkaloids, saponins, total proteins, fats, polyphenols, and antioxidant activity. In addition, the techno-functional properties of the samples were investigated, providing further insights into their potential applications. Overall, these findings contribute to a better understanding of the effects of the applied treatments and represent a relevant step toward the development of PBMAs, a sector that is expected to offer significant opportunities for the food industry in the coming years.

4.1 Growth Kinetics of *Pleurotus Ostreatus*

The results showed a significant increase in glucosamine content throughout the fermentation period for all the pretreatments applied. As expected, this trend is consistent with the progressive growth of the fungus over time, confirming the relationship between N-acetyl-D-glucosamine production (derived from the hydrolysis of chitin, a structural polysaccharide of the fungal cell wall in *P. ostreatus*) and fungal biomass development during fermentation.

The evolution of glucosamine content during the fermentation process is illustrated in Figure 4.1.

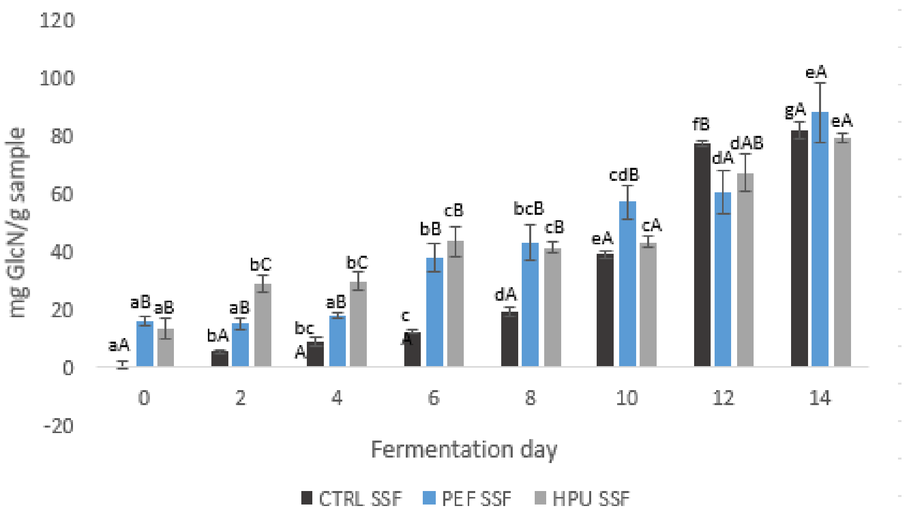


Fig 4.1: *Biomass evolution under three different pretreatments over time. Lowercase letters indicate statistically significant differences (p<0.05) between days within the same treatment, while capital letters indicate statistically significant differences (p<0.05) among treatments on the same day.*

The significantly higher glucosamine content observed at Day 0 for PEF and HPU samples (16.1 and 13.5 mg/g, respectively), compared to the CTRL ones (0.78 mg/g), is not attributable to fungal growth, as the fermentation had just been inoculated. Instead, this suggests that physical pretreatments induced a structural breakdown of the substrate matrix. This degradation likely enhanced the extractability of amino-sugars or released interfering compounds (such as soluble sugars or proteins) that reacted with the Ehrlich reagent, resulting in a higher baseline value compared to the untreated sample.

It is crucial to highlight the significant differences in glucosamine accumulation observed between Day 2 and Day 8. The breakdown of the substrate matrix, caused by the PEF and HPU pretreatments, facilitated the immediate release of essential nutrients (such as fermentable sugars and bioavailable nitrogen) required for fungal metabolism (Galván-D'Alessandro and Carciochi, 2018). Consequently, the typical lag phase observed in the untreated SSF was effectively bypassed, leading to a more efficient and accelerated fermentation process in the early stages.

In the final stage of fermentation, the results obtained for all treatments tended to converge. This suggests that, given sufficient time, the fungus in the control sample is eventually able to reach its maximum biomass potential, matching the yields of the pretreated samples. Therefore, the primary advantage of applying PEF and HPU is not an increase in the absolute final yield, but rather a significant optimization of the growth kinetics, reducing the overall time required to achieve full colonization. At the end of the process, all the samples showed an important increase in glucosamine content, as represented in Table 4.1.

Table 4.1. *Glucosamine content in the first and last day of fermentation for each treatment.*

	DAY 0 [mg GlcN/g dry sample]	DAY 14 [mg GlcN/g dry sample]
CTRL SSF	0.775 ± 1.3	81.7 ± 2.9
PEF SSF	16.1 ± 1.5	88.2 ± 9.5
HPU SSF	13.5 ± 3.3	79.3 ± 1.4

Considering the statistical analysis, the two-way ANOVA revealed that both fermentation time and pretreatment significantly influenced the GlcN content. Moreover, a significant interaction between these two factors was observed ($p < 0.05$). The one-way ANOVA analyses allowed the identification of homogeneous groups within each factor. When considering the effect of fermentation time within each pretreatment, the CTRL fermentation showed the highest variability across the fermentation days. This behaviour may be attributed to the absence of pretreatments, whereas in the PEF and HPU samples the initial opening of the plant cellular structures likely facilitated the early growth of the microorganism, followed by a slight decrease in growth over time. When comparing pretreatments within the same fermentation day, the results obtained for PEF and HPU fermentations were very similar. This may indicate that both pretreatments had a comparable efficiency in enhancing the release of nutritional compounds from the plant matrix, thus supporting fungal growth.

Considering the growth kinetics, it was important to evaluate the behaviour of the trends on a logarithmic scale and to compare them with the typical trend of microbial growth in a batch system, as shown in Figure 4.2.

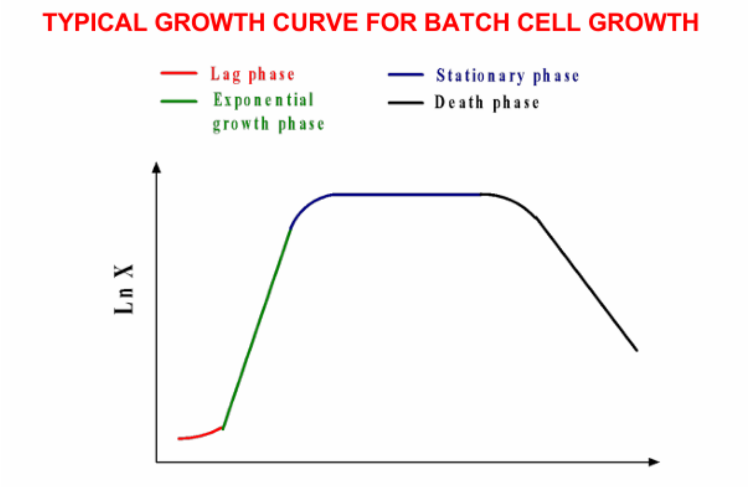


Fig 4.2: *Spilimbergo,2025. Slides of Food and Bioprocess Technologies. University of Padua Kinetic growth of microorganisms in a batch system*

To evaluate whether the experimental results were consistent with the typical microbial growth trend in a batch system (as shown in Figure 4.2), the data was analysed on a logarithmic scale. Specifically, day 0 was excluded from the linear regression analysis in order to better isolate the exponential growth phase. Exponential growth phase of *P. ostreatus* is shown in Figure 4.3.

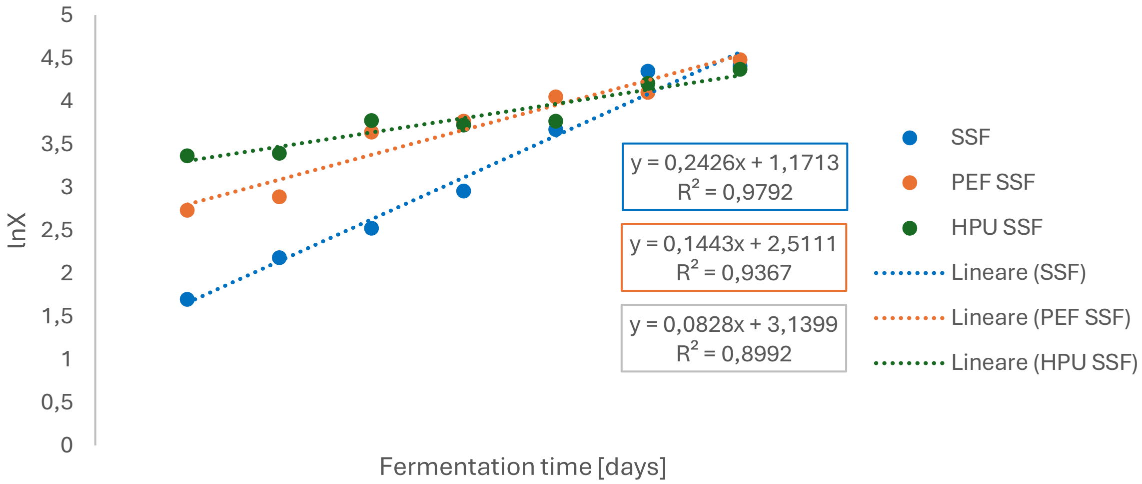


Fig 4.3: *Exponential growth phase of P.ostreatus with different pretreatments*

The linear regressions obtained, where the R^2 values close to 1 for all the treatments, suggest that the system is operating in the exponential phase, where the specific growth rate reaches its maximum value (μ_{max}). Under these conditions, the microbial mass balance mainly depends on the intrinsic growth of the microorganism rather than on substrate availability (i.e., lupine seeds).

In this phase, the exponential growth rate is given by the Equation 4.1. The results obtained are showed in Table 4.2.

$$lnX = \ln X_0 + \mu_{\max} t \tag{4.1}$$

Table 4.2. *Specific growth rate in the exponential phase for each SSF.*

	CTRL SSF	PEF SSF	HPU SSF
$\mu_{\max}$ $[day^{-1}]$	0.2426	0.1443	0.0828

Pre-treatments led to a lower $\mu_{\max}$ compared to the CTRL SSF, likely due to the release of antinutritional compounds from the cellular membrane into the matrix via electroporation and cavitation. These compounds interfere with fungal growth, thereby reducing its growing rate.

However, based on the available data, it was not possible to clearly identify all the typical phases of microbial growth. Therefore, a more comprehensive kinetic analysis would require a wider time range in order to capture the full growth curve.

4.2 Total proteins content

Protein content analysis was performed by an external laboratory using the Dumas method; however, only CTRL and PEF samples were analysed, as HPU samples could not be included within the project timeline. Therefore, only a comparison between fermentation without pretreatment and fermentation with PEF pretreatment was possible. The total behaviour is shown in Figure 4.4.

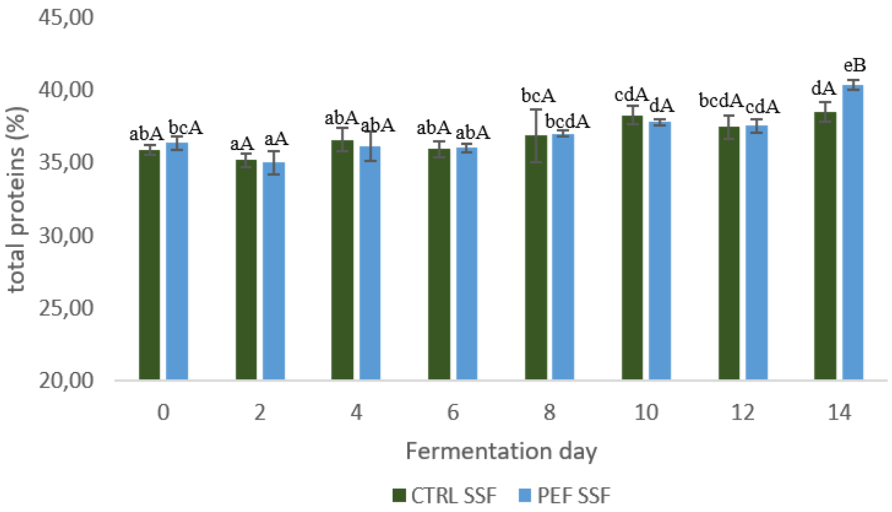


Fig 4.4: *Total proteins content for CTRL and PEF fermentations. Lowercase letters indicate statistically significant differences ($p<0.05$) between days within the same treatment, while capital letters indicate statistically significant differences ($p<0.05$) among treatments on the same day.*

Both fermentations showed a slow growth trend from day 0 to day 14. In particular, both the processes had very similar values until the last day, where PEF achieved a higher final content (Table 4.3).

Table 4.3: *Total proteins content in the first and last day of fermentation for each treatment.*

	DAY 0 [g total proteins/100g dry sample]	DAY 14 [total proteins/100g dry sample]
CTRL SSF	35.9 ± 0.33	38.5 ± 0.68
PEF SSF	36.4 ± 0.48	40.3 ± 0.31

Considering the literature, this increasing trend can be attributed to a fungal biomass accumulation and the conversion of carbohydrates into microbial protein, as reported in lentil flour fermented with *P. ostreatus* (García *et al.,* 2020). In this project, similar increases in protein content have been reported in lentils (+23%) mainly due to fungal growth and conversion of carbohydrates into microbial protein.

However, not all the literature is entirely consistent; some studies have reported a decrease in total protein content following SSF with *P. ostreatus*, likely due to the fungus metabolizing proteins as alternative sources of carbon and energy (Ayllón-Parra et al., 2025). Probably the main effects obtained by the fermentation, and then the enhanced results obtained with the PEF pretreatment, can be attributed more to the decrease in anti-nutritional compounds respect to a real increase in total proteins content.

Statistical analysis confirmed the trends observed in the plot. Considering the uppercase letters, no significant differences were observed between CTRL SSF and PEF SSF on the same day, except for day 14, where PEF fermentation showed a statistically different behaviour. Instead, the results showed in lowercase letters suggest a generally similar trend over time for both treatments, with limited significant variations among days.

4.3 Alkaloids content

One of the main hypotheses of this project concerned the alkaloid content in the vegetable matrix, particularly those belonging to the quinolizidine alkaloid family. In “sweet” species such as *L. albus*, the alkaloid content is significantly lower than in other lupin species and is typically below 1.1 g per 100 g of dry seeds (Carvajal-Larenas et al., 2013).

This relatively low alkaloid concentration allowed the debittering step, commonly applied as a pretreatment when processing lupin seeds, to be avoided. Eliminating this step could reduce the overall processing time of the fermentation, limit excessive water consumption, and minimize the significant solid losses (and consequently nutrient losses) that are typically associated with the debittering process.

First, the results confirmed the low content of quinolizidine alkaloids present in the samples at day 0. Based on the quantification of the reference compound lupanine, all fermentation conditions showed concentrations below the previously reported threshold at day 0. The measured values were $0.038 \pm 0.001\ g/100g$ for CTRL SSF, $0.038 \pm 0.001\ g/100g$ for PEF SSF, and $0.035 \pm 0.002\ g/100$ g for HPU SSF.

The results in terms of lupanine equivalents are reported in Figure 4.5.

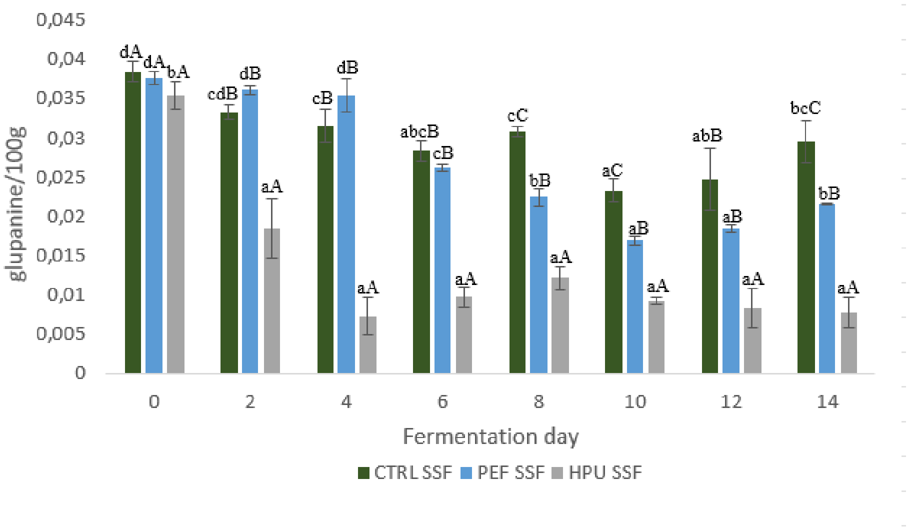


Fig 4.5: *Alkaloids content for three different pretreatments over time. Small letters indicate statistically significant differences (p<0.05) between days within the same treatment, while capital letters indicate statistically significant differences (p<0.05) among treatments on the same day.*

All the fermentations showed a decrease in alkaloid content over time. This suggests that the metabolic activity of the fungi may contribute to the degradation of these toxic compounds, likely through the production of enzymes capable of transforming alkaloids. Some studies on the biological detoxification of plant matrices through solid-state fermentation have reported similar results. For example, fermentation with fungi such as *Rhizopus oligosporus* has been shown to significantly reduce quinolizidine alkaloids in lupin substrates through microbial metabolism and enzymatic activity. These microorganisms are able to produce several enzymes, including proteases, lipases, glycohydrolases and other hydrolytic enzymes, which may participate in the transformation of alkaloid molecules through reactions such as hydroxylation and hydrolysis (Ortega-David, E., & Rodríguez-Stouvenel, A. (2013)).

Although these mechanisms have been mainly described for other filamentous fungi, it is reasonable to hypothesize that similar metabolic pathways could also occur during fermentation with *P. ostreatus*, which is known to produce a wide range of extracellular enzymes involved in the degradation of complex organic compounds.

It is important to note the behaviour observed in fermentations with PEF and, especially, HPU pretreatments. For both, a higher decrease in alkaloid content compared to the CTRL fermentation was observed. Although similar alkaloid contents were observed at the initial fermentation stage, PEF and HPU pretreatments may have increased the accessibility of these compounds by disrupting plant cellular structures. This structural modification likely enhanced the interaction between alkaloids and microbial enzymes during fermentation, leading to a greater degradation in the following days. Results of first and last days of fermentation are written in Table 4.4.

Table 4.4: *Alkaloids content in the first and last day of fermentation for each treatment.*

	DAY 0 [g lupanine/100g dry sample]	DAY 14 [g lupanine/100g dry sample]
CTRL SSF	0.0385 ± 0.0012	0.0296 ± 0.0027
PEF SSF	0.0376 ± 0.0083	0.0217 ± 0.00011
HPU SSF	0.0354 ± 0.0018	0.00775 ± 0.0019

Considering the statistical analysis, both the fermentation days and the pretreatments are very influential parameters for the results trend of the process ($p < 0.05$). Also, the interaction between the two parameters really influenced the behaviour.

The homogeneous groups identified by Tukey's HSD test highlighted different behaviours among the treatments. When considering the effect of fermentation time within the same pretreatment (lowercase letters), the HPU fermentation showed the lowest variability across the fermentation days. This suggests that, after the initial release of alkaloids from the plant cellular structures, HPU pretreatment may have promoted a more efficient conversion of these toxic compounds by microbial enzymes. As a result, after the second day the alkaloid content remained relatively stable during the subsequent fermentation days.

When considering the effect of pretreatment within the same fermentation day (uppercase letters), CTRL and PEF fermentations showed similar behaviour up to day 8. From this point onward, the PEF treatment appeared to promote a greater degradation of alkaloids compared to the control fermentation.

4.4 Saponins

Saponins are another class of compounds that are usually removed from the vegetable matrix with the debittering treatment. One of the aims of this study is to understand if the fermentation process could contribute to reducing the concentration of these antinutritional compounds.

First, the results shown an important difference in saponins concentration at day 0 between the CTRL fermentation and those subjected to PEF and HPU. While the CTRL exhibited low saponin levels, higher initial concentrations were observed in the PEF and HPU-treated samples. This difference may be explained by the disruption of plant cellular structures during the pretreatment step, caused by electroporation in the case of PEF and cavitation in the case of HPU, which likely enhanced the release of saponins from the seed matrix. Results in terms of oleanolic acid equivalents are shown in Figure 4.6.

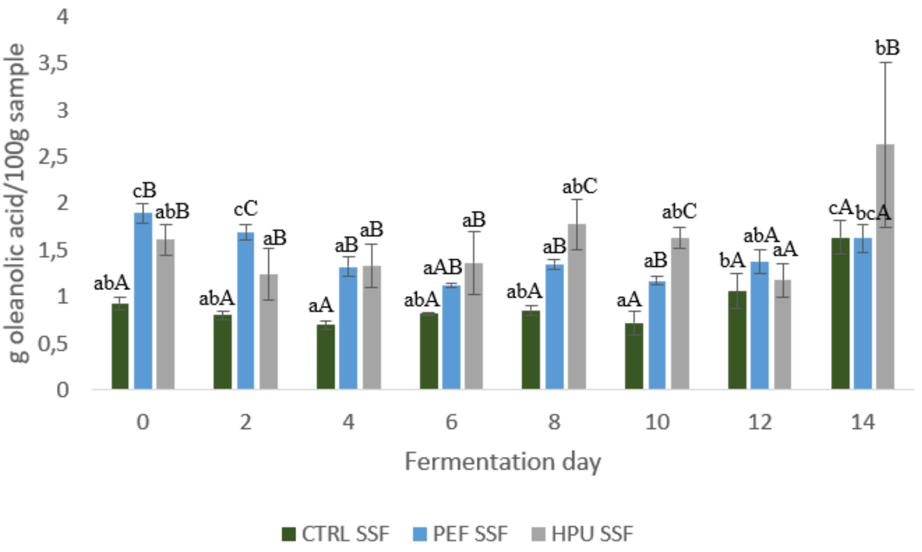


Fig 4.6: *Saponins content for three different pretreatments over time. Small letters indicate statistically significant differences (p<0.05) between days within the same treatment, while capital letters indicate statistically significant differences (p<0.05) among treatments on the same day.*

All the samples showed a similar trend in saponin concentrations over the fermentation time. In contrast to alkaloids, which are known to be extensively degraded during processing, saponins exhibited only limited variations. This behaviour is consistent with previous studies on legumes, where saponins were reported to be less affected by fermentation and processing compared to other antinutritional compounds (Shimelis and Rakshit, 2007).

The increase observed in the last days of fermentation likely does not reflect a real increase in saponin content. Instead, it may be explained by the relative stability of these compounds respect to the others of the matrix: as other compounds are consumed or degraded during fermentation, the saponins remain relatively unchanged, leading to an apparent increase in their concentration when expressed per 100 g of dry sample (Table 4.5).

Table 4.5: *Saponins content in the first and last day of fermentation for each treatment.*

	DAY 0 [g/100g dry sample]	DAY 14 [g/100g dry sample]
CTRL SSF	0.925 ± 0.065	1.63 ± 0.17
PEF SSF	1.89 ± 0.11	1.62 ± 0.15
HPU SSF	6.85 ± 0.02	2.62 ± 0.88

Considering the statistical analysis, two-way ANOVA revealed that both fermentation time and pretreatments significantly affected the results ($p < 0.05$). In addition, a significant interaction between time and pretreatment was observed ($p < 0.05$).

Analysis of homogeneous groups revealed a relatively constant behaviour of oleanolic acid content, particularly during the central part of the fermentation period for all treatments, confirming the stability of these compounds over time. When considering the effect of pretreatment within the same fermentation day (uppercase letters), PEF and HPU fermentations shown a similar trend until the day 8. From this point onward, the higher saponin concentrations observed in HPU fermentation may indicate a more efficient degradation of other antinutritional compounds, compared to the PEF and, in particular, to the CTRL fermentations.

4.5 Fat content

Fat content analysis is one of the most important indicators for evaluating the potential use of lupin seeds as PBMAs, as it provides information on the total amount of this essential macronutrient. Lipids contribute not only to the nutritional value of the product, but also to its technological properties, influencing texture, mouthfeel, and water/oil retention in food formulations. Therefore, monitoring fat content in fermented lupin seeds provides valuable insight into the effects of fermentation on both nutritional and functional quality. Results are shown in

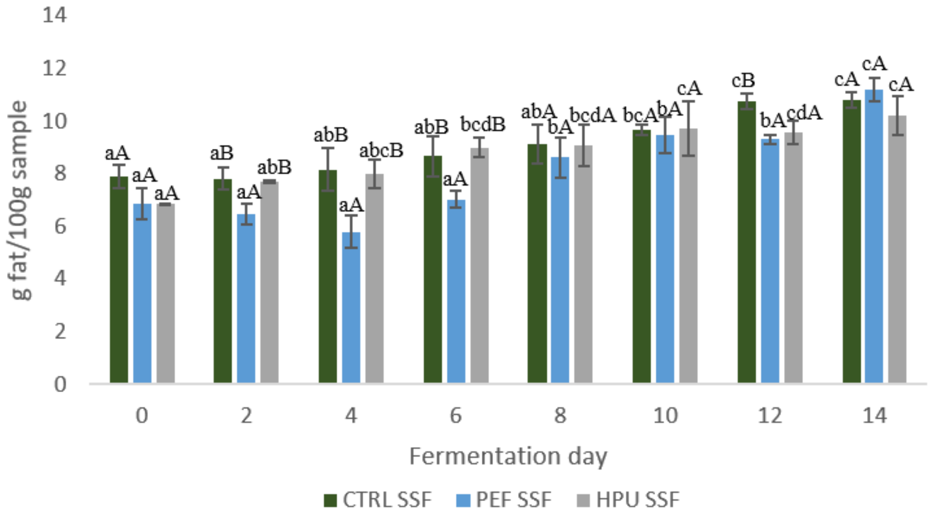


Fig 4.7: *Fat content for three different pretreatments over time. Small letters indicate statistically significant differences (p<0.05) between days within the same treatment, while capital letters indicate statistically significant differences (p<0.05) among treatments on the same day.*

While PEF and HPU treatments caused significant structural changes through electroporation and cavitation, these physical alterations did not impact the fat content measured at Day 0. This is likely because the high efficiency of petroleum ether as a solvent ensures a complete extraction of lipids from the vegetable matrix, regardless of the pre-treatment applied. In fact, the results at day 0 showed very similar values, confirming that all treatments belong to the same homogeneous statistical group.

For each fermentation it's possible to observe a growing trend throughout the days. This increase in fat concentration may be explained by the microbial preferences for the biological growth: during fermentation, microorganisms like *P. ostreatus* primarily consume carbohydrates (starches and soluble sugars) and some proteins as their energy source. In this way, this consumption and this loss of dry matter can leads to a relative enrichment of lipids in the sample (Zhang *et al.*, 2022). Furthermore, the moderate increase in fat content could be partially attributed to microbial lipid synthesis, although this contribution is generally considered minor. The increase in fat content is shown in Table 4.6.

Table 4.6: *Fat content in the first and last day of fermentation for each treatment.*

	DAY 0 [g/100g dry sample]	DAY 14 [g/100g dry sample]
CTRL SSF	7.89 ± 0.46	10.8 ± 0.31
PEF SSF	6.88 ± 0.59	11.2 ± 0.47
HPU SSF	6.85 ± 0.017	10.2 ± 0.75

Statistical analysis confirmed this trend: when evaluating the effect of time within the same treatment, several homogeneous groups were identified throughout the fermentation process. Although a general increasing trend was detected via One-Way ANOVA and Tukey's HSD test, many daily values remained statistically comparable, particularly during the intermediate stages. Furthermore, when comparing the effects of pre-treatments on the same day a consistent behaviour across the different fermentation trials was observed. In this context, the application of PEF and HPU did not significantly alter the fat content yields. his suggests that the exhaustive nature of petroleum ether extraction overrides any structural changes induced by the pre-treatments at the beginning of the process.

4.6 Polyphenols and Antioxidant Activity

Unlike alkaloids and saponins, polyphenols are not primarily considered antinutritional compounds, but rather bioactive molecules with antioxidant properties. Antioxidant activity is an important quality indicator of fermented foods, as antioxidant compounds may neutralize reactive oxygen species, contributing to the prevention of diseases related to oxidative stress. The potential issues related to these compounds is associated to the digestibility of the fermented food: some phenolic compounds, particularly tannins, may produce antinutritional effects by interacting with proteins and digestive enzymes, potentially reducing protein digestibility. Therefore, for this reason it's important to evaluate their concentration and then the nutritional quality of the lupine-based substrate during fermentation. Results are shown in terms of galic acid equivalents in Figure 4.8.

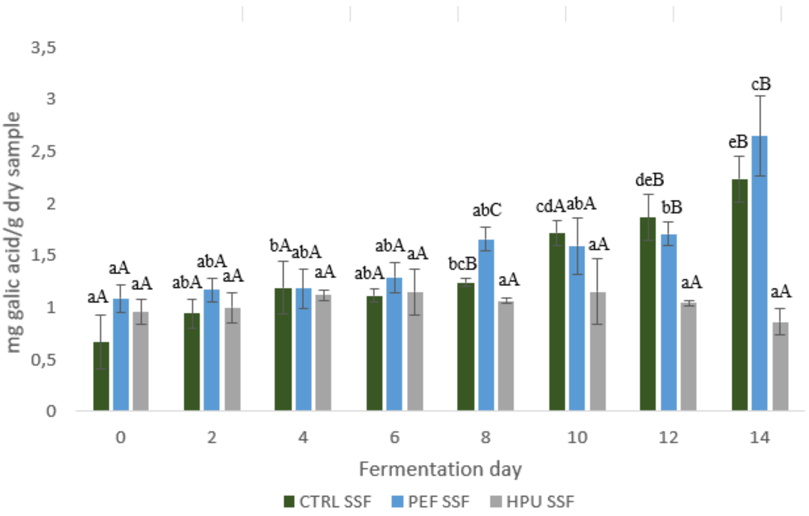


Fig 4.8: *Polyphenols content for three different pretreatments over time. Small letters indicate statistically significant differences (p<0.05) between days within the same treatment, while capital letters indicate statistically significant differences (p<0.05) among treatments on the same day.*

First, the results obtained shown the effect of the electroporation and of the cavitation on the release of polyphenols content: the use of pretreatments allowed a release of these compounds from the vegetable membrane to the matrix of the lupine seeds. In this way, a higher concentration of polyphenols for PEF and HPU fermentations was relieved respect to the CTRL one (Table 4.7).

Table 4.7: *Polyphenols content in the first and last day of fermentation for each treatment.*

	DAY 0 [mg galic acid/g dry sample]	**DAY 14 [mg galic acid/g dry sample]**
CTRL SSF	0.662 ± 0.25	2.23 ± 0.23
PEF SSF	1.08 ± 0.13	2.64 ± 0.11
HPU SSF	0.951 ± 0.12	0.854 ± 0.12

Regarding the overall trend, a general increase in polyphenol concentration was observed, which continued until the final day of fermentation. This upward trend during the fermentation period is consistent with other scientific literature, such as Olukomaiya *et al.* (2020). In their study, the fermentation of lupin flour was conducted using fungi of the genus Aspergillus (*Aspergillus sojae*, *Aspergillus ficuum*, and their co-cultures); in that case as well, the fermentation process promoted an increase in free polyphenols within the matrix. The increase in Total Phenolic Content (TPC) can be attributed to the enzymatic activity of the fungi. Cellulolytic and hemicellulolytic enzymes produced during fermentation are able to partially degrade plant cell wall structures, facilitating the release of phenolic compounds that are originally bound to the cell wall matrix.

This trend is not observed in HPU fermentation. This phenomenon could be attributed to the high energy of acoustic cavitation, which may have induced the water sonolysis within the medium.

The resulting formation of hydroxyl radicals ($OH\cdot$) likely led to the partial oxidative degradation of phenolic compounds into quinones or other oxidized derivatives. Consequently, these reactions may have reduced both the stability and the extractability of polyphenols throughout the 14 days of fermentation. his hypothesis is supported by literature; specifically, Wang *et al.* (2020) demonstrated a direct correlation between hydroxyl radical concentration and the degradation of polyphenols. In this context, the sonochemical effect acts as a secondary, degradative force that opposes the beneficial mechanical effects induced by cavitation. Thus, the stagnant or constant behaviour observed during HPU fermentation is scientifically consistent.

Statistical analysis revealed that both fermentation time and the type of pre-treatment were significant factors ($p < 0.05$) affecting polyphenol content. Furthermore, a significant interaction between these two variables was observed. Regarding inter-day variability, the HPU fermentation displayed the highest number of homogeneous groups, with lowercase letters confirming a lack of significant differences across most days. For all treatments, a similar trend was maintained until Day 10; beyond this point, the emergence of non-homogeneous groups indicated a sharp and significant increase in TPC for CTRL and PEF fermentations.

Another approach used to evaluate the antioxidant potential of lupin seeds involves the direct quantification of total Antioxidant activity. This measurement considers the contribution of both polyphenols and other bioactive compounds present in the matrix.

The results obtained in terms of Trolox equivalents shown an interesting analogy with the polyphenols behaviour, where a general increase in Antioxidant activity was observed for CTRL and PEF fermentations, while a stability trend is recorded for the HPU fermentation. Results are shown in terms of mg of Trolox in Table 4.8 and Figure 4.9.

Table 4.8: *Antioxidant activity in the first and last day of fermentation for each treatment.*

	DAY 0 [mg trolox/g dry sample]	DAY 14 [mg trolox/g dry sample]
CTRL SSF	0.844 ± 0.058	1.31 ± 0.049
PEF SSF	0.726 ± 0.046	1.77 ± 0.42
HPU SSF	0.559 ± 0.022	0.782 ± 0.064

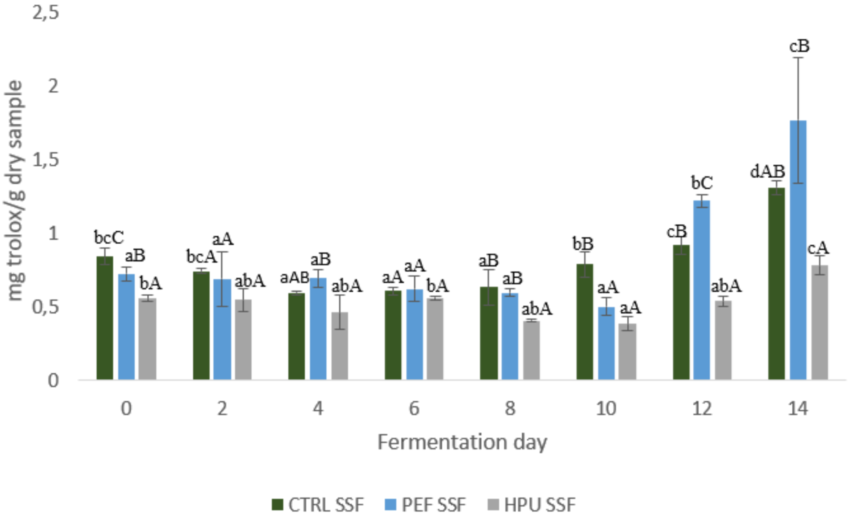


Fig 4.9: *Antioxidant activity for three different pretreatments over time. Small letters indicate statistically significant differences (p<0.05) between days within the same treatment, while capital letters indicate statistically significant differences (p<0.05) among treatments on the same day.*

Considering the statistical analysis, the two-way ANOVA confirmed that both pretreatment and fermentation time significantly affected the results ($p < 0.05$), as well as their interaction.

The statistical analysis highlighted a relatively constant trend for each treatment up to day 10. From this point onward, CTRL and PEF fermentations showed an increasing trend, as confirmed by the lowercase letters, which indicate statistically significant differences among days within the same treatment. In contrast, HPU samples maintained the same homogeneous group throughout the fermentation period, confirming a more stable behaviour over time. When considering comparison between treatments, CTRL and PEF exhibited similar trends along the days.

The observation of the same trends in these different analyses strengthens the hypothesis of the progressive enzymatic activity of the fungi acting on the cellular matrix during the fermentation, facilitating the release of phenolic and antioxidant compounds. Furthermore, the relatively constant trends observed for both polyphenol content and antioxidant activity in HPU fermentation support the hypothesis of the secondary effect of ultrasounds on the cellular membrane of lupine seeds. The cavitation phenomenon, in addition to the enhancing the release of compounds from the membrane into the matrix, may also generate sonochemical effects that can promote the oxidation or degradation of phenolic and antioxidant compounds.

4.7 Techno-functional analysis

4.7.1 Soluble proteins

Total protein content is considered a nutritional parameter, as it reflects the overall protein availability in the sample. In contrast, protein solubility is related to the physicochemical properties of proteins and is therefore included among techno-functional characteristics, as it influences properties such as emulsification, foaming, and gelation behaviour.

Soluble proteins evaluation is very important in fermented products because it provides an idea of protein structural changes and of their digestibility. As reported in Olukomaiya *et al.* (2020), fermentation processes influence the dimensions of the proteins: the presence of enzymes as proteases, allows the degradation of the long chains of proteins in smaller compounds, thereby reducing the molecular weight of the sample proteins.

This degradation could increase their solubility exposing their hydrophilic groups and reducing their molecular size. In this way, proteins could be more accessible and digestibility could be enhanced.

Results are shown in Figure 4.8.

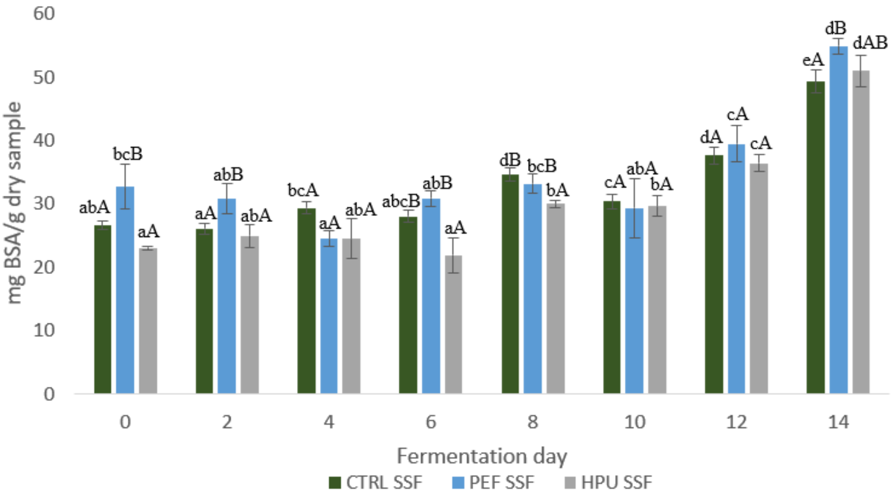


Fig 4.10*: Antioxidant activity for three different pretreatments over time. Small letters indicate statistically significant differences (p<0.05) between days within the same treatment, while capital letters indicate statistically significant differences (p<0.05) among treatments on the same day.*

The results confirmed the intense enzymatic activity occurring mainly in the last days of the process.

After a first period of stability in soluble protein content for all the samples, a significant growing trend is observed from the day 12. This increase is primarily attributed to the proteolytic degradation of high-molecular-weight protein chains into smaller, more soluble peptides.

At the end of the process, all the samples revealed an increase in the total amount of soluble proteins, as shown in table 4.9.

Table 4.9: *Soluble protein content in the first and last day of fermentation for each treatment.*

	DAY 0 [mg BSA/g dry sample]	DAY 14 [mg BSA/g dry sample]
CTRL SSF	26.5 ± 0.63	49.3 ± 1.8
PEF SSF	32.7 ± 3.5	54.9 ± 1.3
HPU SSF	23.0 ± 0.28	50.9 ± 2.5

Considering the differences between the different pre-treatments, CTRL and HPU maintained a similar trend throughout all the fermentation period, while the PEF fermentation shown higher values, in particular at the beginning and at the end of the process. This superiority may be attributed to the higher efficiency of electroporation, which might have facilitated the release of intracellular proteins from the cellular membrane to the matrix. In this way, a more efficient transformation of complex proteins into smaller, soluble peptides compared to the other treatments.

Considering statistics analysis, the two-way ANOVA revealed both fermentation time and the type of pre-treatment were significant factors ($p < 0.05$) affecting soluble proteins content, as well as their interactions. For each treatment, homogenous groups are observed over the central days of fermentation, confirming the stability of soluble protein content until the last days of the process.

4.7.2 Gelation capacity

Based on the gelation capacity analysis, samples corresponding to the most relevant time points for each treatment were analysed: day 0, day 10, and day 14. CTRL and PEF fermentations showed similar results, while the SSF with the HPU treatment showed higher values (12-18%). Results are shown in table 4.10.

Table 4.10: *Gelation capacity at day 0, day 10 and day 14 for each treatment.*

	CTRL SSF [%]	PEF SSF [%]	HPU SSF [%]
DAY 0	14	8	18
DAY 10	8	10	12
DAY 14	8	8	18

The ability of proteins to form three-dimensional networks that trap liquid is the basis of gelation. This process takes place when globular proteins lose their native structure and unfold, facilitating the interaction of molecular groups that form aggregates, leading to a final gel structure. (Ayllón-Parra e*t al.*, 2025)

In the present study, no significant differences in LGC were observed among the different fermentation times for any of the treatments (CTRL, PEF, and HPU), suggesting that SSF did not markedly affect the gelation properties over time. However, the HPU treatment exhibited consistently higher LGC values compared to the other treatments, starting from day 0.

In contrast with the studies of Ayllón-Parra e*t al.* (2025), who reported a marked increase in LGC for chickpea, oat and *Chlorella vulgaris with P. ostreatus,* and a slight decrease for quinoa, in our study the fermentation time had little effect on LGC in samples subjected to pretreatment (PEF &HPU), whereas in the CTRL samples, LGC decreased by 6%. These results suggest that the type of vegetable matrix strongly influences this parameter, reflecting the native structure of globular proteins in plant-based sources.

In the case of PEF fermentation, a lower LGC is observed at the day 0 respect to the other treatments. As reported in the literature (Taha *et al.*,2022) PEF processing can induce controlled protein unfolding and increase the exposure of reactive groups such as sulfhydryl (–SH) which can enhance protein–protein interactions and, under appropriate conditions, improve gelation properties. Indeed, PEF has been shown to exposing functional groups that facilitate network formation. However, when structural modifications are too extensive or lead to excessive aggregation, the capacity of proteins to form a stable three-dimensional gel network may be compromised, resulting in poorer gelation performance.

In the case of HPU fermentation, the higher LGC values may be explained by uncontrolled protein denaturation induced by the treatment, which negatively affected the techno-functional property. Additional studies are necessary to better evaluate the effects of pretreatments on protein denaturation and gelation capacity throughout the fermentation period across all treatments.

4.7.3 Water absorption Capacity and Oil absorption Capacity

Water and oil absorption capacities (WAC and OAC) are key functional properties that are commonly evaluated to assess the technological performance of PBMAs.

These parameters provide insight into the ability of the matrix to retain water and interact with lipids, thereby influencing texture, stability, and mouthfeel. High WAC values are associated with improved moisture retention, while OAC plays a crucial role in the incorporation and stabilization of lipid components within the food matrix.

The results, expressed as grams per gram of dry sample, are presented in Table 4.11 and 4.12.

Table 4.11: *Water absorption capacity at day 0, day 10 and day 14 for each treatment.*

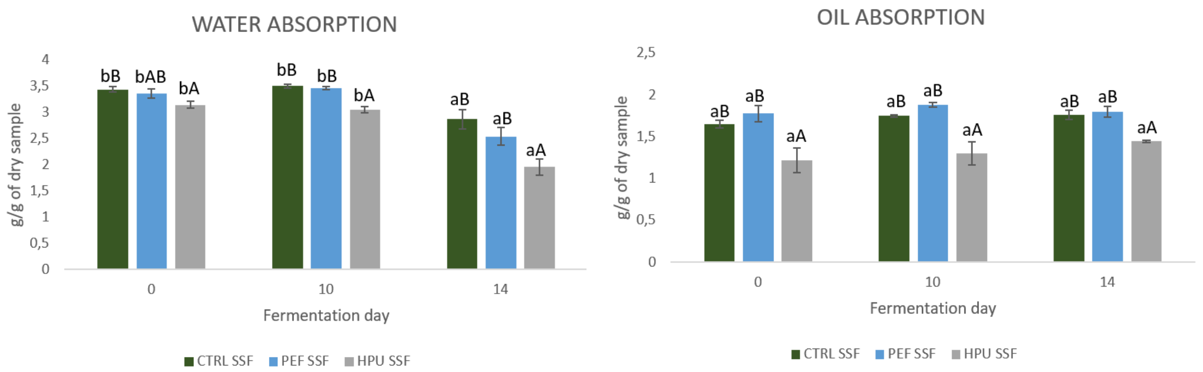
	CTRL SSF [g/g dry sample]	PEF SSF [g/g dry sample]	HPU SSF [g/g dry sample]
DAY 0	3.44 ± 0.055	3.36 ± 0.085	3.15 ± 0.070
DAY 10	3.51 ± 0.037	3.47 ± 0.033	3.05 ± 0.064
DAY 14	2.88 ± 0.18	2.54 ± 0.17	1.96 ± 0.15

Table 4.12: *Oil absorption capacity at day 0, day 10 and day 14 for each treatment.*

	CTRL SSF [g/g dry sample]	PEF SSF [g/g dry sample]	HPU SSF [g/g dry sample]
DAY 0	1.65 ± 0.047	1.78 ± 0.099	1.22 ± 0.15
DAY 10	1.75 ± 0.014	1.88 ± 0.028	1.30 ± 0.14
DAY 14	1.76 ± 0.056	1.80 ± 0.066	1.45 ± 0.012

The results showed a slight decrease in WAC on the final day of fermentation for all pretreatments. Regarding treatment effects, both CTRL and PEF fermentations exhibited stable and comparable values up to day 14, when PEF SSF showed a lower value compared to the control. Overall, HPU samples displayed lower WAC values than the other treatments, remaining relatively constant from day 0 to day 10, followed by a decrease at the final time point.

In contrast, oil absorption capacity remained relatively constant across all treatments throughout the fermentation period, although slightly lower values were observed for HPU SSF. The total behaviour and the statistical analysis are represented in Figure 4.11.



(a) (b)

Fig 4.11: *WAC (a) and OAC (b) for three different pretreatments over time. Small letters indicate statistically significant differences (p<0.05) between days within the same treatment, while capital letters indicate statistically significant differences (p<0.05) among treatments on the same day.*

Statistical analysis (one-way ANOVA, $p < 0.05$) confirmed the limited influence of the fermentation period on OAC, as indicated by the presence of homogeneous groups (lowercase letters) within each pretreatment over time. In contrast, uppercase letters highlighted significant differences among treatments at the same fermentation time, with HPU samples consistently showing lower OAC values compared to CTRL and PEF throughout the entire fermentation period.

Regarding WAC, a different behaviour was observed. Lowercase letters indicated a pronounced effect of fermentation time the final stage, where a decrease was evident for all treatments. Additionally, uppercase letters confirmed significant differences among treatments at each time point, with HPU generally exhibiting lower WAC values compared to CTRL and PEF, and a more marked decline at the end of fermentation.

Considering the literature, a decrease in WAC during fermentation was also reported in lupin flour subjected to SSF, where values significantly decreased compared to the unfermented sample (Olukomaiya *et al.*,2020). This decrease in WAC has been attributed to a reduction in hydrophilic groups available to bind water molecules during fermentation, likely due to biochemical modifications of proteins and polysaccharides.

According to Olukomaiya *et al.* (2020), OAC did not show a definitive trend following fermentation. While some samples exhibited a slight increase and others a minor decrease, the results demonstrated a general lack of consistency across the different treatments. This overall stability suggests that the impact of fermentation on OAC is less significant or predictable compared to other functional properties.

Considering the different treatments applied, HPU is the one more different in WAC and OAC results. These lower results obtained at the last day of fermentation for WAC and along all the fermentation period for OAC, may be attributed to structural modifications induced by ultrasound processing. Although HPU is a non-thermal technology, it can generate localized high temperatures and shear forces due to cavitation, leading to protein denaturation and aggregation phenomena like those observed during thermal treatments. According to Li *et al.* (2023), heat treatment can enhance both WAC and OAC up to a maximum threshold. Beyond this point, prolonged heat treatment leads to excessive protein denaturation and the formation of aggregates, which subsequently causes a decrease in water and oil absorption capacities. In the same way, thermal hotspot in HPU lupine seeds may have led to protein denaturation and to a decrease of these values.

4.7.4 Foaming and Emulsifying Stability

Foaming and emulsifying capacities are also essential techno-functional properties, that play a crucial role in defining the texture, stability, and overall quality of many food products, particularly in aerated and emulsion-based systems.

Foaming capacity was evaluated both immediately after homogenization (F_{t0}) and after 20 minutes (F_{t20}), in order to assess the ability of the foam to be retained over time. The results are expressed as mL of foam per gram of sample and are reported in Table 4.13.

Table 4.13: *Foaming capacity at day 0, day 10 and day 14 for each treatment*

Fermentation day	Treatment	$F_{to}\ [mL/g]$	$F_{t20}[mL/g]$
DAY 0	CTRL	$5,04 \pm 0.087$	0
	PEF	4.91 ± 0.16	0
	HPU	6.91 ± 0.19	0
DAY 10	CTRL	3.74 ± 0.48	0
	PEF	4.84 ± 0.15	0
	HPU	10.9 ± 1.38	3.56 ± 0.22
DAY 14	CTRL	4.88 ± 0.27	1.28 ± 0.046
	PEF	6.06 ± 0.18	1.48 ± 0.095
	HPU	13.5 ± 1.7	3.07 ± 0.43

Based on these results, foaming capacity increased with fermentation time. Moreover, HPU-treated samples consistently showed the highest values at each time point, maintaining a relatively high foam volume even after 20 minutes from homogenization, indicating improved foam persistency.

In addition, foaming stability was evaluated: considering this parameter, all treatments showed a similar trend. All samples, regardless of the pretreatment applied, reported a stability between

25 and 30%, showing a similar stability between the initial foam volumes and those after 20 minutes.

Regarding emulsifying stability, the fermentation period influenced all samples, with a slight decrease in the ESI observed over time. This effect was more pronounced in HPU-treated samples, which showed a marked reduction in ESI at the final day of fermentation, as illustrated in Figure 4.10.

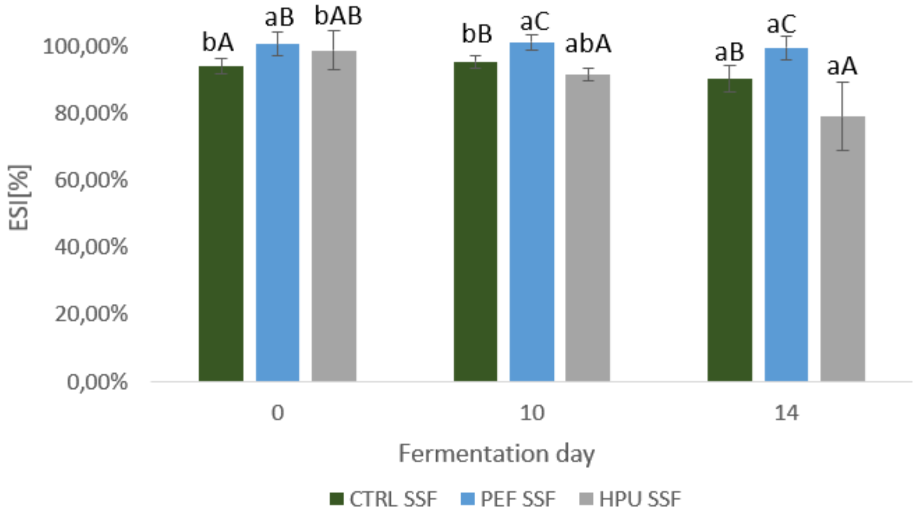


Fig 4.12: *Emulsifying stability index for three different pretreatments over time. Small letters indicate statistically significant differences ($p<0.05$) between days within the same treatment, while capital letters indicate statistically significant differences ($p<0.05$) among treatments on the same day.*

In addition to the marked decrease observed in HPU-treated samples at day 14, which may be attributed to experimental variability (as indicated by the high standard deviation), the results also showed lower ESI in CTRL fermentations compared to PEF samples. Notably, PEF samples exhibited the highest emulsion stability across all fermentation times, maintaining consistently elevated ESI values throughout the entire process (maximum reduction <1%).

From a statistical perspective (one-way ANOVA, $p < 0.05$) uppercase letters indicate significant differences among treatments at the same fermentation time, confirming the superior performance of PEF in terms of emulsion stability. Moreover, the relatively limited variation indicated by lowercase letters suggests that fermentation time had a minor effect on ESI compared to the impact of the pretreatment.

As reported by Taha *et al*. (2022), PEF treatment might be able to enhance the emulsifying properties of proteins by inducing structural modifications. This can be achieved through the improvement of the solubility and partial unfolding of the protein molecules, exposing the hydrophobic groups on the protein's surface. Additionally, PEF may reduce particle size, further contributing to improved interfacial properties and emulsion stability. However, the underlying mechanisms are not yet fully understood, and further studies are needed to clarify the molecular effects of PEF on protein functionality.

Conclusions and future outlooks

The main objective of this thesis project was to evaluate the impact of SSF of *P. ostreatus* applied to lupine seeds as plant-based matrix with the aim of gaining a deeper understanding of how this aerobic process can enhance both the nutritional and antinutritional profile of this PBMA. Furthermore, this study investigated the effect of two pre-treatments technologies, PEF and HPU, applied to improve the fermentation efficiency. In addition, this project was carried out without the conventional debittering step, in order to assess whether fermentation alone could effectively reduce the antinutritional compounds typically removed during debittering.

The results presented demonstrate the important impact of both the pre-treatments and the fermentation period on the final nutritional profile of lupine seeds. Across all the analysis it was observed that electroporation and the cavitation, induced by PEF and HPU respectively, affected the cellular structure of the seeds by increasing membrane permeability and promoting structural disruption; this facilitated the release of intracellular compounds, enhancing their availability for fermentation. Indeed, the study showed that these non-thermal treatments have strong potential to improve bioavailability of sugars, proteins, and bioactive compounds.

Considering the analyses conducted, it was first important to evaluate the effectiveness of fermentation and pre-treatments on the increase of total and soluble proteins within the matrix, which represents a key parameter in the development of PBMAs. An overall increasing trend in total protein content was observed in all samples; however, this effect is likely attributable more to the reduction of anti-nutritional compounds than to an actual increase in protein content. Instead, the results obtained for soluble proteins, which are closely related to protein digestibility, were particularly relevant. An important increase in soluble proteins can be attributed to the fermentation process, and in particular to the activity of some fungal enzymes such as proteases, that allows the breakdown of complex protein structures into smaller peptides, thereby reducing their molecular weight. Furthermore, PEF-treated samples showed a higher increase in soluble proteins compared to the others. This effect may be linked to the greater efficiency of electroporation, which likely enhanced membrane permeability and facilitated the release of intracellular proteins into the surrounding matrix.

Considering the other analyses, one of the most important results regarding the decrease of quinolizidine alkaloids for all the samples investigated. Starting from a “sweet” species such as *L. albus*, which typically contains lower quantities of alkaloids respect to other lupine species (<1. 1g/100g of dry seeds), it was important to confirm the substantial decrease of these compounds throughout the fermentation days, particularly in the PEF- and HPU-treated samples. These pre-treatments may have enhanced the accessibility of alkaloids to fungal enzymes involved in the degradation of these complex organic compounds. However, it is important to note that neither fermentation itself nor the individual pre-treatments (PEF and HPU) significantly affected the content of other potentially toxic compounds, such as saponins, which remained relatively stable throughout the process. For this reason, further investigations are required to evaluate whether the debittering step can be safely avoided, as its removal could improve overall process efficiency by reducing processing time and water-energy consumption. Additionally, future studies on other lupine species would be valuable to better understand the effectiveness of these pre-treatments in matrices characterized by a higher alkaloid content.

Furthermore, important techno-functional considerations can be done. For all samples, the final days of fermentation were associated with lower values of water absorption capacity (WAC), oil absorption capacity (OAC), and emulsifying capacity, while gelation and foaming capacity were only slightly affected. These results suggest that extended fermentation may negatively impact certain functional properties, and further investigations are needed to determine whether shorter fermentation times could optimize these parameters. Regarding the pre-treatments, PEF yielded the most favourable results across all techno-functional analyses.

In conclusion, the present study has provided a comprehensive evaluation of the potential of fermented lupine seeds as accreditable PBMA. highlighted the valuable nutritional profile of this plant matrix and demonstrated how the SSF can enhance these properties. explored a debittering-free approach, obtaining promising results that support the possibility of simplifying the process by avoiding this conventional pre-treatment step. Furthermore, it highlighted the potential effectiveness of technological pre-treatments, in particular PEF, in improving fermentation performance and overall product quality.

However, further investigations are required to optimize both the fermentation time and the technological parameters in order to achieve the best possible nutritional and functional profile. Once these conditions are optimized, a key future perspective will be the scale-up of the process to an industrial level. In this context, the design and implementation of suitable bioreactor systems will be essential to overcome the typical limitations of SSF, particularly those related to heat transfer and oxygen mass transfer in larger and more complex scales.

Nomenclature

C = protein concentration (mg/mL)
F = dilution factor (-)
F_0 = foaming capacity (-)
h = height of the sample (mm)
L = optical path length of the cuvette (m)
PE = weight of extract (g)
PF = final weight of the tube after drainage (g)
PO = weight of extract and tube (g)
P_{50} = proportion of antimicrobials with resistance levels exceeding 50% (-)
rpm = revolutions per minute (rpm)
T = turbidity index (m)
V_{foam} = volume of the foam (mL)
V_{sample} = volume of the sample (mL)
v/v = volume per volume ratio (-)
w/v = weight per volume ratio (g/mL)
w/w = weight to weight ratio (-)
φ = oil volume fraction (-)

Acronyms

AOAC = Association of Official Analytical Chemists
BCA = bicinchoninic acid
CAFOs = Concentrated Animal Feeding Operations
CAGR = Compound annual growth rate
DIAAS = Digestible Indispensable Amino Acid Score
EAI = Emulsifying Activity Index
ESI = Emulsifying Stability Index
FAO = Food and Agriculture Organization
FDA = Food and Drug Administration
FRAP = Ferric Reducing Antioxidant Power
FSI = Foaming Stability Index
GRAS = generally recognized as safe
HPU = High-Power Ultrasounds
IPCC = Intergovernmental Panel on Climate Change
LDL = low-density lipoprotein
LGC = least gelation concentration
LMICs = Low- and middle-income countries

LUC = Land-use change
OAC = oil absorption capacity
PBMAs = Plant-based meat alternatives
PEF = Pulsed Electric Fields
QAs = quinolizidine alkaloids
RCF = relative centrifugal force (expressed in g)
SD = Standard Deviation
SmF = submerged fermentation
SSEA = South and Southeast Asia
SSF = solid-state fermentation
TPTZ = 2,4,6-Tripyridyl-s-triazine
Trolox = 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Tukey’s HSD = Tukey’s Honestly Significant Difference test
TVP = Textured vegetable proteins
UPV = Polytechnic University of Valencia
USD = United States dollar
USDA = United States Department of Agriculture
WAC = water absorption capacity

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