



**CATOLICA**  
**ESCOLA SUPERIOR DE BIOTECNOLOGIA**

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PORTO

**PULSE UP: PROMOTING PULSES IN THE PORTUGUESE  
AGRI-FOOD SYSTEM THROUGH CHILDREN'S DIETS**

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of Ph.D. in  
Science, Food Technology, and Nutrition

*Eyla Darc Ferro Vieira*

June 2024





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June 2024



Dedico a meus filhos Benício e Clarice, minhas maiores fontes de inspiração e força.

**“Se podes olhar, vê. Se podes ver, repara.”**

Jose Saramago

## Resumo

Os padrões alimentares baseados em alimentos de origem vegetal são mais saudáveis e podem diminuir os impactos ambientais. Ao pensar nas fontes de proteína vegetal na dieta, é essencial considerar as leguminosas, que são grandes aliadas das dietas saudáveis e sustentáveis, do prado ao prato. Ao integrar sistemas de cultivo, as leguminosas reduzem as emissões de gases de efeito estufa. Além disso, se substituir a proteína animal em locais de consumo excessivo, as leguminosas têm potencial para mitigar a pressão sobre os recursos naturais. Apesar da função crítica na sustentabilidade dos sistemas alimentares a área de cultivo de leguminosas na Europa diminuiu nos últimos sessenta anos, e o consumo é de cerca de 2 a 8 kg por pessoa por ano, quando a recomendação da FAO (Food and Agriculture Organization) é de 15 a 25 kg por pessoa por ano. Diante deste baixo consumo, e tendo em conta todos os benefícios, a identificação de estratégias e políticas para a promoção de leguminosas no sistema agroalimentar Europeu deve estar entre as principais prioridades dos decisores políticos. No entanto, implementar mudanças requer muitas alterações em diversos setores. Assim, tomar medidas direcionadas a grupos específicos, como crianças, pode produzir resultados mais assertivos. O principal objetivo desta tese é desenvolver e validar soluções práticas e viáveis para promover as leguminosas no sistema agroalimentar Português através da alimentação infantil. Primeiramente, foi realizada uma revisão crítica da literatura sobre as medidas que devem estimular o consumo de leguminosas na alimentação infantil. Considerando a viabilidade e impacto, duas destas medidas foram selecionadas para serem desenvolvidas. As ações escolhidas foram enriquecer um alimento infantil com leguminosas e a promoção das leguminosas no ambiente escolar. Em relação ao enriquecimento do alimento com leguminosas, o primeiro estudo desenvolvido ajudou a selecionar a leguminosa mais adequada para ser adicionada ao produto. Em seguida, um iogurte infantil enriquecido com tremço foi desenvolvido e caracterizado em termos nutricionais, reológicos e sensoriais, e demonstrado seu impacto ambiental. Também foram apresentadas as alterações metabólicas do produto. Em relação à promoção de leguminosas no ambiente escolar, foi elaborado um material de orientação para professores do pré-escolar sobre a promoção do consumo de leguminosas, realizado um grupo focal *online* para caracterizar o consumo de leguminosas em jardins de infância selecionados, e avaliar o material produzido. Os resultados desta tese indicam que o enriquecimento de iogurtes infantis com tremço e a promoção de leguminosas em jardins de infância são

medidas viáveis que podem aumentar a demanda e o consumo de leguminosas. Enriquecer iogurtes infantis com tremço é uma alternativa para produzir um produto nutritivo e possivelmente funcional, ao mesmo tempo em que pode impulsionar uma cadeia de valor local baseada em leguminosas. Adicionalmente, a promoção de leguminosas nos jardins de infância, através de compras locais e ações que estimulem o consumo dos pré-escolares, podem favorecer a produção nacional, com inclusão social e económica, e ajudar a incrementar o hábito de consumo de leguminosas na população Portuguesa.

**Palavras-chave:** Alimentação infantil; Jardins de infância; Leguminosas; Sustentabilidade.

## **Abstract**

Plant-based eating patterns are healthy and can decrease environmental impacts. When discussing sources of dietary plant protein, it is essential to consider the dry-harvested legume crops, known as pulses. Pulses are farm-to-fork actors that promote sustainability and healthy diets. When integrating cropping systems, pulses reduce greenhouse gas emissions per unit area. Also, if animal protein is replaced in places of overconsumption, pulses can potentially mitigate the pressure on natural resources. Despite the critical function in food systems' sustainability, the area growing pulses in Europe declined in the last sixty years, and consumption is around 2 to 8 kg per person-year when the Food and Agriculture Organization (FAO) recommendation is 15 to 25 kg per person-year. In the face of this low consumption and given all benefits, identifying strategies and policies for promoting pulses in Europe's agri-food system should be among policymakers' top priorities. However, implementing changes requires many alterations in several sectors. Thus, taking specific measures directed to priority groups, such as children, may produce more assertive results. This thesis's main objective is to develop and validate practical and viable solutions to promote pulses in the Portuguese agri-food system through children's diets. First, a critical literature review on the measures that should stimulate pulses in children's diets was proceeded. Considering the feasibility and impact, two actions were selected to be developed. The chosen actions were to enrich a child's food with a pulse and pulse promotion in the school food environment. Regarding enriching children's diets with pulses, the first study helped select the pulse that is more suitable for developing the product. Next, a children's yogurt enriched with lupin was developed and characterized in nutritional, rheological, and sensorial terms, and environmental impact was demonstrated. Also, metabolomic alterations were presented. Concerning pulse promotion in the school food environment, a guidance material for preschool teachers on promoting the consumption of pulses was prepared, and an online focus group was carried out to characterize the consumption of pulses in selected kindergartens and evaluate the material produced. The results in this thesis indicate that the enrichment of children's yogurts with lupin and the promotion of pulses in kindergartens are viable measures that can increase the demand and consumption of pulses. Enrich children's yogurt with lupin is an alternative to produce a nutritious and perhaps functional product while it may drive a local pulse-based value chain. Additionally, promoting pulses in kindergartens by local procurement and actions that stimulate preschoolers' consumption can favor domestic production with social and

economic inclusion and help increment the habit of pulse consumption in the Portuguese population.

**Keywords:** Child's food; Kindertartens; Pulses; Sustainability.

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**PART I.**  
**Introduction**

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## **Chapter 1. General Introduction**

This chapter will contextualize the importance of pulses for the sustainability of European agri-food systems and discuss strategies to promote their consumption in children's diets. Also, a review of pulses' benefits and recommendations in childhood will be provided, and the thesis' objectives, scope, and outline will be presented.

Some parts of this chapter were taken from the following peer-reviewed publications:

Vasconcelos MW, Gomes AM, Pinto E, Ferreira H, **Vieira EDF**, Martins AP, et al. 2020. The Push, Pull, and Enabling Capacities Necessary for Legume Grain Inclusion into Sustainable Agri-Food Systems and Healthy Diets. In: *Hidden Hunger and the Transformation of Food Systems: How to Combat the Double Burden of Malnutrition?* Edited by: Hans Konrad Biesalsk.

**Vieira EDF**, Gomes AM, Gil AM, Vasconcelos MW. 2021. Pulses' Benefit in Children's Diets: A Narrative Review. *Journal of Obesity and Chronic Diseases* 5(1):13-22.

**Vieira EDF**, Gomes AM, Gil AM, Vasconcelos MW. 2021. The Transition towards Sustainable Diets Should Encourage Pulse Consumption in Children's Diets: Insights for Policies in Food Systems. *Journal of Obesity and Chronic Diseases* 5(2): 43-51.

## 1.1 Introduction

Global food systems are operating with excessive consumption of natural resources while delivering poor health outcomes (Caron *et al.*, 2023). The way food is produced, processed, packaged, transported, commercialized, and consumed is among the leading causes of environmental degradation and climate change. The current food production is responsible for utilizing around 40% of arable land and 70% of global freshwater and is responsible for up to 30% of global anthropogenic GHG emissions (Gibbs and Cappuccio, 2022). Additionally, expanding agricultural land use contributes to deforestation and biodiversity loss (Rockström *et al.*, 2020; Raven and Wackernagel, 2020). Still, the predominant food pattern from these systems is associated with the increased availability of low-nutrient, highly processed foods and animal protein-rich diets that have favored obesity and chronic diseases. At the same time, undernutrition persists in the developing world (Willett *et al.*, 2019). The coexistence of nutritional issues and climate changes, interacting with each other, and sharing social drivers has been termed Global Syndemic (Dietz and Pryor, 2022). Changing food systems to benefit human nutrition and health while protecting the environment is one of the biggest challenges for the next 30 years (Fanzo *et al.*, 2021).

Sustainable diets can be synthesized as those that ensure healthy food for all, now and in the future, while not compromising the environment (Triches, 2021). Achieving sufficiently sustainable food systems is complex, involving the promotion of measures such as re-orientation in agricultural priorities, adopting food production practices that protect the environment, prioritizing short and seasonal food chains, advances in governance, and reducing food loss and waste. In addition, it is necessary to promote dietary changes that increase the demand for healthy foods with less environmental impact. Dietary patterns must be considerably modified in Europe and other developed regions to reduce ecological pressure (Willett *et al.*, 2019; HLPE Report, 2017).

Data has shown that plant-based eating patterns are healthy and can decrease environmental impacts, especially in reducing GHG emissions and chemical fertilizer applications (Springmann *et al.*, 2018). To adequately feed a growing world population without further degrading natural resources and worsening climate change, the EAT-Lancet commission evaluated nutritional and environmental evidence to suggest a healthy diet from sustainable food production, calling it the 'Planetary-health diet.' Accordingly, around 60% of daily protein intake should be plant protein (Woolston, 2020). Adopting the EAT-Lancet diet will demand a considerable change in western diets, with a rise of 100% in plant foods consumption and a reduction of 50% in red meat and sugar (Willett *et al.*, 2019).

When discussing sources of dietary plant protein, it is essential to consider the dry-harvested legume crops, known as pulses. Pulses are farm-to-fork actors that promote sustainability and healthy diets (Rawal and Navarro, 2019), being strong contributors to solving global food security challenges (Ngigi *et al.*, 2022). Unlike other crops, pulses have a capacity for symbiotic atmospheric nitrogen fixation, which permits a more sustainable cultivation system by reducing synthetic nitrogen fertilizers. This self-sufficiency represents an environmental advantage since synthetic nitrogen fertilizers lead to GHG emissions (Yanni *et al.*, 2024) and globally are a significant source of eutrophication (Fanzo *et al.*, 2021). Also, pulse cultivation increases soil carbon sequestration, which means partial absorption of atmospheric CO<sub>2</sub> while promoting soil biodiversity, recovering or improving soil fertility, and requiring less water than other crops (Yanni *et al.*, 2024).

These benefits are available when pulses are included as a component of sustainable cropping systems (intercropping, crop mixtures, conservation agriculture, agroforestry, and others). In these contexts, pulses often enhance yield, reduce pests and diseases, and increase the food production systems' resilience (Yanni *et al.*, 2024). When pulses are integrated into cropping systems, GHG emissions can be up to seven times less per unit area than other crops (Stagnari *et al.*, 2017). Besides, if replacing animal protein in places of overconsumption, pulses have the potential to mitigate the pressure on natural resources, such as land and water, and reduce GHG emissions associated with livestock management (Cusworth *et al.*, 2021).

The European Green Deal Farm to Fork strategy for a fair, healthy, and environment-friendly food system aims to contribute to the Europe union's climate neutrality by 2050. It focuses on a common agricultural policy (CAP) that increases the participation of organic farming, reducing GHG emissions and chemical pesticide use by 50%, and synthetic fertilizer use by 20%, compared with 1990 levels, by 2030 (European Commission, 2021). In this context, pulse's introduction to European agri-food systems could help the achievements of the Green Deal. A recent study compared the environmental advantages of pulse introduction in crop rotation in three climatic zones in Europe. The results pointed out that the introduction of pulses and other grain legumes into conventional cereal and oilseed rotations delivered human nutrition at a lower environmental cost, mainly due to the reduction of synthetic nitrogen fertilizers requirements (Costa *et al.*, 2021).

Despite the critical function in food systems' sustainability, the area growing pulses in Europe declined from 4.7% of arable land in 1961 to below 2% (Cusworth *et al.*, 2021). In 2014, European farmers grew grain legumes on only 1.6% of the arable land, compared

to 14.5% worldwide (Costa *et al.*, 2021; Reckling *et al.*, 2016). Pulses are often rejected by the consumers and insufficiently used by farmers, caterers, and retailers (Vasconcelos *et al.*, 2020). After World War II, European farm practices and their business models became dependent on high-yielding and intensive systems for cereals, oilseed, dairy, pork, and beef production. The focus on improving chemical inputs, cultivars, and machinery has led to considerable improvements in the yields of cereals and oilseeds but made pulses' production financially untenable and socially undesirable (Cusworth *et al.*, 2021).

The FAO estimates an average world pulse consumption of 7 kg per person per year when the recommendation should be 15 to 25 kg per person-year (McDermott and Wyatt, 2017; FAO Trade and Markets Division, 2016). Among European countries, the highest consumption is observed around the Mediterranean, with a consumption of 3 to 8 kg per person-year, while in Northern Europe, the consumption is less than 2 kg per person-year (kalogeropoulos *et al.*, 2010). According to food availability data, in Portugal, the average consumption is 4.9 kg per person-year (INE, 2021). In comparison, a consumption survey indicates that the Portuguese consume an average of 18 g of legumes daily, with children having the lowest consumption (Lopes *et al.*, 2017).

In the face of this low consumption and given all benefits, identifying strategies and policies for promoting pulses in Europe's agri-food system should be among policymakers' top priorities. However, implementing such changes is not simple, requiring many alterations in several sectors (FAO and WHO, 2019). Thus, taking specific measures directed to priority groups may be particularly important to produce more assertive results (HLPE Report, 2017). Since food preferences develop early in childhood, this is a suitable period to encourage healthy, sustainable diets (Hollis *et al.*, 2020) and pulse consumption. The primary nutritional advantage of pulses consumption in children's diets is to help achieve daily intake recommendations, especially for micro-nutrients (Jager *et al.*, 2019). Implementing actions across the food system to promote pulses consumption by children could significantly positively impact health and the environment (HLPE Report, 2017; Szczybyło *et al.*, 2020). It is time to discuss feasible and impact strategies to promote pulses in Portuguese children's diets, favoring healthy and sustainable food systems.

## **1.2 Pulses benefits in nutrition, health, and sustainability**

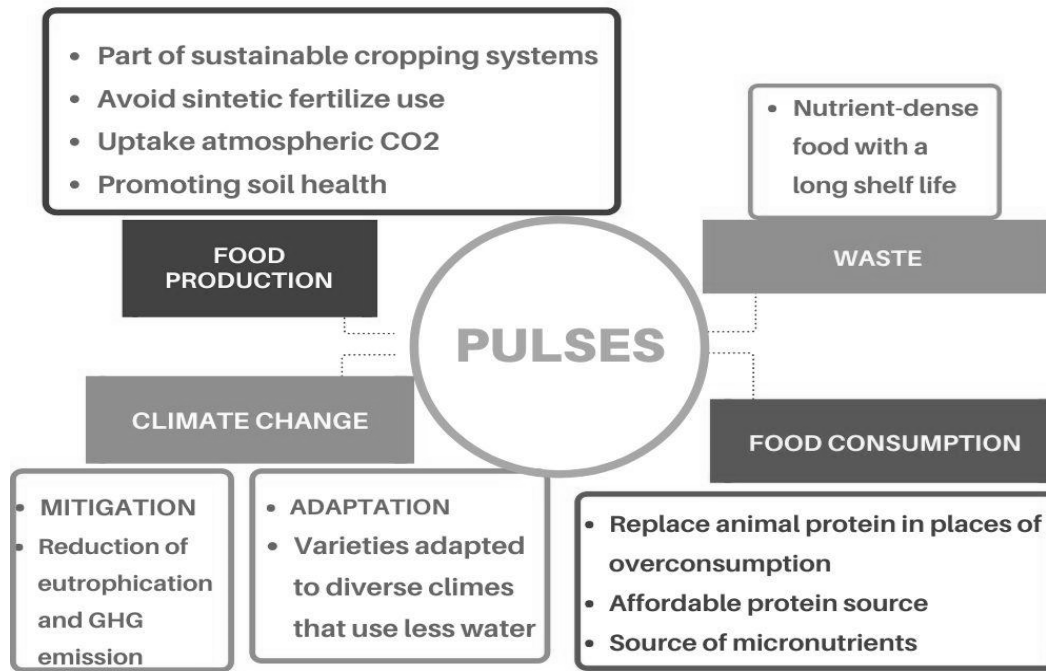
Legume grains are an extremely nutritious food that belongs to Leguminosae (or Fabaceae) family. They produce pods with edible seeds for humans and animals. The term "pulses" is used to denominate a subgroup of dry-harvested legume crops with low-fat

content, such as beans, lentils, chickpeas, broad beans, and lupines. It excludes legumes that are eaten fresh (e.g., green peas) or used for oil production (e.g., soybean) (FAO, 1994). The words "legumes" and "pulses" can be partially interchangeable since all pulses are considered legumes (Singh, 2017).

Legumes are one of the first plants in the world to be domesticated. Humans have cultivated these foods as early as 10,000 years ago (Vasconcelos *et al.*, 2020), growing in diverse parts of the world in different conditions, from hot to cold climates. Their vast geographical range allowed pulses were included across cultures and cuisines worldwide, giving rise to many delicious dishes on all continents (FAO OCCP, 2016).

Pulses deliver multiple advantages to the sustainability of food systems, contributing to balancing the deficit in plant protein production in many areas of the world, including Europe (Stagnari *et al.*, 2017). As a source of proteins, fiber, and bioactive compounds, it has increasing importance in improving human health (Marinangeli *et al.*, 2020). Also, due to the capacity to fix atmospheric nitrogen pulses are highly suitable for inclusion in low-input cropping systems. In agroecosystems, they help to mitigate greenhouse gas emissions and break the cycles of pests and diseases (Stagnari *et al.*, 2017). Figure 1.1 explains how pulses can favor sustainable food systems.

Pulses are amongst the most nutritionally valued crops on the planet (Kouris-Blazos and Belski, 2016). With low energy density and a small quantity of fat, pulses are rich in proteins, fibers, minerals, complex B vitamins, bioactive carbohydrates, and phytochemicals (Yanni *et al.*, 2024). Carbohydrates are a significant component of legume seeds (60-65%). Starch is the most abundant carbohydrate, but simple sugars are also present. Although in large quantities, the carbohydrates in pulses are slowly digested due to the considerable amounts of dietary fiber (Singh, 2017; Berrios *et al.*, 2010). Dietary fiber includes four main components: indigestible oligosaccharides, soluble fiber, insoluble fiber, and resistant starch. Resistant starch corresponds to a considerable fraction in pulses due to two main factors: the high proportion of amylose to amylopectin and the cell walls in whole grains, which consist of a barrier to the action of starch's digestive enzymes, even after cooking (Brummer *et al.*, 2015). These factors explain the low glycemic index of pulses. Fat content is usually low in pulses, but it has significant mono- and polyunsaturated fatty acids (Yanni *et al.*, 2024).



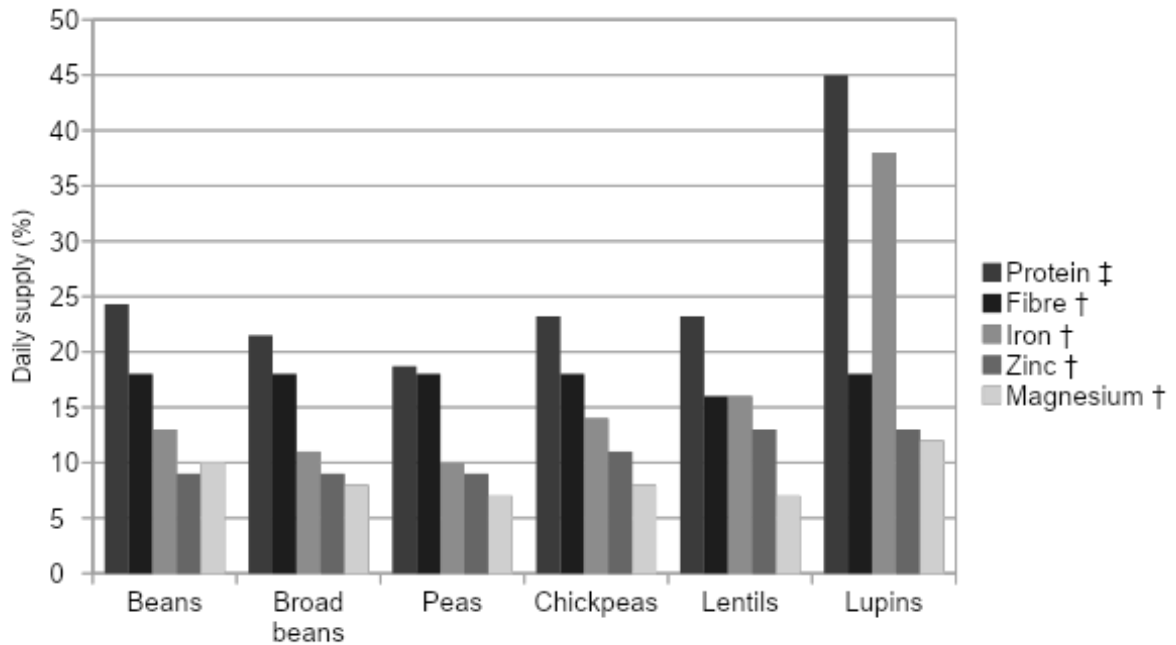
**Figure 1.1** The role of pulses in shaping sustainable food systems

In general, pulses range from 20% to 35% (dry weight) of protein (Grdeń and Jakubczyk, 2023). There is an imbalance in pulses' amino acid composition. Lysine is present in higher amounts, whereas the sulfur amino acids methionine and cysteine are available in limited quantities. Since pulses lack these essential amino acids, they have been considered lower-quality protein sources than animal protein (Havemeier *et al.*, 2017). However, pulses are frequently consumed with cereals, which have high sulfur amino acid levels that complement amino acid profiles forming a high biological value. Accordingly, traditional meals worldwide include pulses and cereal combinations, making excellent complete protein sources (Iriti and Varoni, 2017).

In developing countries, where animal protein is inaccessible for part of the population, pulses (combined with cereals) represent an inexpensive source of dietary protein. In developed countries, pulses could partially replace animal proteins, providing environmental and health gains (Yanni *et al.*, 2024). Harwatt *et al.* (2017) predicted that if the American population substituted beef for beans in a hypothetical situation, considering equivalent calories and proteins, the US could reach up to 74% of the 2020 GHG reduction target. Therefore, pulses are an affordable and environmentally friendly source of protein (Sahruzaini *et al.*, 2020) that can fit in different socioeconomic contexts.

Due to their nutritional richness, pulses may be significant in periods of raised nutritional demand like childhood. Figure 1.2 illustrates the importance of pulse

consumption to meet dietary demands in children’s diets. It is possible to observe that only two tablespoons (50g) of cooked pulses can provide about 20% of daily protein requirements, 18% of fiber requirements, and about 10% of the requirements of iron, zinc, and magnesium for preschool children (4-6 years old).



**Figure 1.2** Percent (%) coverage of daily supply of protein, fiber, and minerals by consuming 50g of cooked pulses\*

\*Based on the Portuguese food composition table for cooked pulses.

‡ Percentage of daily supply (% DS) calculated based on Europe dietary reference for protein in 6 years old children.

† Percentage of daily supply (% DS) calculated based on Europe dietary reference for minerals in children aged 4-6 years.

Unlike other nutrient-dense foods such as fruit, vegetables, and animal protein, pulses have storage advantages over fresh products, staying wholesome over a long shelf-life (Sahruzaini *et al.*, 2020) without cold chain requirements. It makes pulses suitable for the reality of low-income settings and represents a substantial benefit in terms of food waste (Sahruzaini *et al.*, 2020; Lartey *et al.*, 2016), one of the significant problems of the current food systems.

As if it were not enough, increasing evidence points to a significant role of pulses in preventing and controlling frequent diseases worldwide such as obesity, diabetes, cardiovascular diseases, and cancer (Margier *et al.*, 2018). Epidemiological and intervention studies indicated beneficial results on cardiovascular risk factors, body composition,

inflammatory profile, and quality and metabolism of gut microbiota (Ferreira *et al.*, 2020; Ramdath *et al.*, 2016).

In terms of glucose regulation, the pulses' benefits seem to be precise. A systematic review and meta-analysis of randomized controlled experimental trials revealed that pulses, alone or in low-glycemic-index diets or high-fiber diets, favor biomarkers of glycemic control in adult humans (Sievenpiper *et al.*, 2009). The effect of pulses on glycemic control has been attributed to the type and amount of undigested carbohydrates present in pulses, such as fiber, resistant starch, and oligosaccharides that reduce the glucose absorption rate. In addition, pulses may promote an insulin-sparing mechanism that improves medium to longer-term glycemic control (Sievenpiper *et al.*, 2009).

In recent years, childhood diabetes has risen in many countries, with an annual increase estimated at around 3% (Patterson *et al.*, 2014). The regular consumption of legume grains may be an effective alternative to control glycemic response in diabetic children, but few studies have investigated this question. A survey carried out with a group of insulin-dependent diabetic children (21 children with ages between 7-16 years old) offered four different diets in random order. Among the four diets, three only differed in fiber content (High fiber: 2-8 g of fiber/100 calories; Medium fiber: 0-8 g of fiber/100 calories; Low fiber: 0-3 g of fiber/100 calories; and Beans: 1-5 g of fiber/100 calories), and one was different in fiber and protein content because it contained soybeans as a fiber source. The children took capillary blood samples 30, 60, and 120 minutes after breakfast and the mid-morning snack. It was observed that the soybean diet produced better glycemic control, with more stable blood glucose levels over time (Baumer *et al.*, 1982).

Regular pulses consumption has also been related to lower body weight and obesity risk. Several human randomized intervention studies have reported that pulses consumption increases satiety, decreases food intake, and reduces body weight (Clark and Duncan, 2017). Growing evidence indicates that pulses can potentially modify gut hormone production and consequently alter appetite (Ferreira *et al.*, 2020; Nilsson *et al.*, 2013).

Few studies have investigated the association between pulses consumption and body weight in children. A cross-sectional study examined the relationship between plant food intake (including pulses) and body mass index (BMI) in different nations. It was observed that adolescents (13–14 years old) who consumed plant foods at least three times a week, and children (6–7 years old) who consumed vegetables three or more times a week, had a more reduced BMI than those who were never eating plant-based foods (Wall *et al.*, 2018). Accordingly, including pulses and other plant foods in diets would impact the prevention of

childhood obesity. Similar results have been found in studies relating to children's body weight and adherence to the Mediterranean diet. There is a substantial body of evidence pointing out that the adoption of a Mediterranean-like dietary pattern, which includes regular pulse consumption, is associated with lower body weight independently of other factors like age, sex, socioeconomic status, and physical activity (Mistretta *et al.*, 2017; Pereira-da-Silva *et al.*, 2016; Tognon *et al.*, 2014).

Pulses are highly nutritious foods capable of supplying much of the nutritional demands in childhood, improving nutrition with a low environmental cost. Evidence points out that pulses' regular consumption may also reduce childhood obesity and be an effective alternative to control glycemic response in diabetic children.

### **1.3 The non-nutritive compounds**

Even though pulses possess valuable nutritional composition, they contain non-nutrient compounds that can potentially reduce the bioavailability of nutrients or produce gastrointestinal discomfort. Nevertheless, depending on the dose consumed, these substances can also have bioactivity, exerting positive metabolic effects on the human body (Margier *et al.*, 2018; Sánchez-Chino *et al.*, 2015). Non-nutrient compounds can be broadly classified into two groups: proteinaceous compounds and non-proteinaceous compounds. Table 1.1 presents the main non-nutrient compounds in pulses.

In the proteinaceous group are inhibitors of digestive enzymes (amylase and proteases) and lectins, usually found in common beans (*Phaseolus vulgaris*). The inhibitors of digestive enzymes may have antinutritional properties, but there is little evidence of deleterious effects in humans (Silva and Silva, 2000). Their protein constitution can be easily disabled by thermal treatment (Gilani *et al.*, 2012). On the other hand, *in vitro*, *in vivo*, and human studies have revealed that the Bowman-Birk family (BBI) protease inhibitors can play a protective or suppressive role against intestinal inflammation and colorectal cancer (Clemente and Olias, 2017). A randomized, double-blind, placebo-controlled trial in patients with active ulcerative colitis suggested that a BBI concentrate extract from soy achieved benefit in clinical response and remission induction concerning placebo (Lichtenstein *et al.*, 2007).

Lectins are compounds capable of binding to specific receptors on the surface of intestinal cells, which can cause toxicity or interfere with nutrient absorption (Petroski and Minich, 2020). However, there is no evidence that lectins in properly processed foods (cooking at a temperature around 100°C) are harmful to humans (Roos *et al.*, 2013).

Additionally, lectins have shown antimicrobial and antitumor activity *in vitro* (Lagarda-Diaz *et al.*, 2017). Essays demonstrated that plant lectins have antiviral properties against coronaviruses and the acquired immunodeficiency syndrome (AIDS) virus (He *et al.*, 2018; Keyaerts *et al.*, 2007).

In the non-proteinaceous group, the undesirable effects are related to the decreasing bioavailability of minerals and proteins, the production of bitter or astringent flavors, and gastric discomfort. Saponins, alkaloids, phytic acid, phenolic compounds, and  $\alpha$ -galactosides make up this group. Fortunately, the reduction of these compounds may be achieved by usual processing such as dehulling (mechanical removal of the seed coat), soaking, washing, germination, fermentation, or heat processing – either alone or in combination (Singh *et al.*, 2017a; Fabbri and Crosby, 2016; Roos *et al.*, 2013; Martínez-villaluenga, *et al.*, 2008).

Saponins and alkaloids are compounds involved in the plant defense mechanisms, usually found in the seed coats of pulses, mainly in lupins (*Lupinus spp*), chickpeas (*Cicer arietinum*), common beans (*Phaseolus vulgaris*), and broad beans (*Vicia faba*). They confer a bitter taste and are considered anti-nutrients due to their toxicity to humans. In contrast, saponins and alkaloids appear to have anti-mutagenic, hypoglycemic, hypocholesterolemic, hepatoprotective, immunomodulatory, and antioxidant activities in experimental animal studies and *in vitro* assays (Kouris-Blazos and Belski, 2016). Removal of these compounds can be achieved by soaking, followed by cooking. The immersion water must be discharged (Singh *et al.*, 2017a; Kouris-Blazos and Belski, 2016; Michaelsen *et al.*, 2009).

**Table 1.1** Main non-nutrient compounds in pulses, potentially beneficial and anti-nutritive effects, and strategy to reduce them

<b>Non-nutrient compounds</b>	<b>Legume grain where it is most found</b>	<b>Potential health benefit</b>	<b>Potential anti-nutritive effect</b>	<b>Strategy for reduction/elimination</b>	<b>References</b>
<b>Protein nature:</b>					
Lectins	<i>Phaseolus vulgaris</i>	Anti-carcinogenic and antimicrobial effects	Gut wall damage, intoxication	Heat processing ( $\geq 100^{\circ}\text{C}$ )	He <i>et al.</i> (2018); Lagarda-Diaz <i>et al.</i> , (2017); Roos <i>et al.</i> (2013)
Amylase inhibitors	<i>Glycine max</i> <i>Phaseolus vulgaris</i>	Reduction of blood glucose and blood cholesterol	Inhibition of digestive enzymes	Heat processing, fractionation	Rebello <i>et al.</i> , (2014); Gilani <i>et al.</i> , (2012)
Protease inhibitors	<i>Glycine max</i> <i>Phaseolus vulgaris</i>	Anti-carcinogenic and anti-inflammatory effects	Inhibition of digestive enzymes	Heat processing, fractionation	Clemente and Olias (2017); Rebello <i>et al.</i> , (2014); Gilani <i>et al.</i> , (2012)
<b>Non-protein nature:</b>					

Phytic acid	<i>Phaseolus vulgaris</i> <i>Vigna unguiculata</i> <i>Vicia faba</i>	Antioxidant, anti-carcinogenic, prevention of renal stone, reduction of blood glucose and blood cholesterol	Decrease intestinal absorption of proteins, iron, calcium, and zinc	Mechanical removal of the seed coat, germination, soaking, fermentation	Singh <i>et al.</i> (2017b); Singh (2017); Michaelsen <i>et al.</i> (2009); Schlemmer <i>et al.</i> (2009)
Phenolic compounds	<i>Vicia faba</i> <i>Vigna angularis</i> <i>Lens culinaris</i>	Antioxidant, anti-inflammatory, antimicrobial, and analgesic activities	Decrease intestinal absorption of proteins, iron, calcium, and zinc	Mechanical removal of the seed coat, soaking in a salt solution, and cook	Singh <i>et al.</i> (2017b); Singh <i>et al.</i> (2017c); Fabbri and Crosby (2015)
Saponins	<i>Cicer arietinum</i> <i>Phaseolus vulgaris</i> <i>Pisum sativum</i> <i>Lupinus spp</i>	Anti-mutagenic, hypocholesterolemic, anti-inflammatory, and antioxidant activities	Decrease intestinal absorption of iron and zinc	Soaking, germination, and cooking	Singh <i>et al.</i> (2017a); Roos <i>et al.</i> (2013)
Alkaloids	<i>Vicia faba</i> <i>Lupinus spp</i> (non-domesticated species)	Anti-mutagenic, anti-inflammatory, and antioxidant activities	Toxic effects	Soaking overnight and cooking	Kouris-Blazos and Belski, 2016

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$\alpha$ -galactosides	<i>Lupinus spp</i> <i>Vigna unguiculata</i>	Prebiotic effect	Reduction of protein bioavailability, flatulence	Soaking in water or saline solution, fermentation and germination	Martínez-Villaluenga <i>et al.</i> (2008)
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Phytic acid is a significant compound with an antinutritional effect present in pulses. Phytates chelate multivalent metal ions and form insoluble compounds with several nutrients, particularly iron, zinc, and calcium (Singh, 2017; Singh *et al.*, 2017b). It is evident that phytate markedly decreases iron absorption, even in small amounts (Roos *et al.*, 2013). Iron plays an essential role in infant brain development (McCann *et al.*, 2020). Therefore, high amounts of phytate may be detrimental to infant development. Phytate content can be reduced by applying food-processing methods such as mechanical removal of the seed coat, germination, and fermentation, minimizing phytate by up to 90%. Soaking is another traditional processing method that can also reduce the phytate in legumes by about 20% (Roos *et al.*, 2013; Michaelsen *et al.*, 2009).

In recent years, a valuable characteristic of phytate has been explored in experimental and *in vitro* studies, such as antioxidant and anticancer activities and positive effects on blood glucose and cholesterol levels. Phytate also has a notable role in preventing kidney stones, as observed in animal models, observational studies, and clinical trials (Schlemmer *et al.*, 2009).

Phenolic compounds and their derivatives are the most studied bio-active in pulses. Present mainly in the hull, they are highly oxygenated molecules with an aromatic ring with one or more hydroxyl substituents, which confer antioxidant properties. The health benefits of pulses are partially due to the antioxidant characteristics of their phenolic compounds (Sánchez-Chino *et al.*, 2015; Singh *et al.*, 2017c; Perlatti *et al.*; 2016). On the other hand, polyphenols, mainly tannins, may have antinutritional properties for binding and reducing the absorption of iron, other metals, and proteins. However, it is unknown if this can be a problem in children. Some condensed tannins have an astringent flavor, which can collaborate with the rejection of foods with higher tannin levels. Tannins are thermostable and, therefore, thermal treatment does not significantly decrease their content. Immerse in a salt or sodium bicarbonate solution before domestic cooking cuts the tannin content by 37.5% to 77.0%. The immersion water must be discharged (Michaelsen *et al.*, 2009; Fabbri and Crosby, 2016).

Pulses (especially lupins) are also the highest amounts of oligosaccharides. Among the oligosaccharides, the  $\alpha$ -galactosides raffinose, stachyose, and verbascose are the most abundant (Singh *et al.*, 2017b; Ma *et al.*, 2017). The  $\alpha$ -galactosides are not digested by humans' gastrointestinal tracts, are fermented by anaerobic bacteria, and produce gases. That is why legume grain ingestion is associated with flatulence, bloating, or gastric discomfort.

Moreover, depending on the doses,  $\alpha$ -galactosides can implicate protein utilization (Fan *et al.*, 2015; Martínez-villaluenga *et al.*, 2008).

In contrast, oligosaccharides have shown a potential prebiotic effect, increasing several groups of bacteria responsible for normobiosis of the colon (Gullón *et al.*, 2015). It has been proposed that a 3 g/day dose of  $\alpha$ -galactosides is sufficient to obtain health advantages without digestive problems. Soaking in water or saline solution with bicarbonate is the most efficient treatment for partial elimination of  $\alpha$ -galactosides from grain (Brummer *et al.*, 2015). Fermentation and germination are methods for sharply reducing the  $\alpha$ -galactosides content (Martínez-villaluenga *et al.*, 2008).

### *1.3.1 Are non-nutrients beneficial or risky for children?*

Infants and young children with a high growth velocity can be sensitive to the antinutritional effect of non-nutrients compounds (Michaelsen *et al.*, 2009). The impact of these compounds on nutritional status will depend on diet quality, dose consumed, and growing demand. In a well-balanced diet, non-nutritional substances should not represent a risk. However, these substances can reduce mineral bioavailability in an unbalanced diet, compromising the food's nutritional value (Margier *et al.*, 2018; Schlemmer *et al.*, 2009), especially for infants or malnourished children (Roos *et al.*, 2013; Michaelsen *et al.*, 2009). As processing eliminates or reduces the non-nutrients, antinutritional factors usually only manifest if the seed or the flour is consumed raw (López-Martínez *et al.*, 2017). Thus, it is crucial to give special attention to processing when these vulnerable groups consume pulses. Under adequate handling to reduce antinutritional factors, it is possible to include pulses in the complementary feeding. In several parts of the world, pulses compose the complementary infant diet, being well-tolerated and well-accepted by children (Trehan *et al.*, 2015).

Pulses are generally consumed after some treatment, and the most common is soaking, followed by cooking (boiled as soup). Fermentation or germination also are critical processes usually used before consumption. These treatments are expected to alter the composition, increase the bioavailability of the nutrients, and improve the taste of pulses (Kalogeropoulos *et al.*, 2010; Martínez-villaluenga *et al.*, 2008). Thus, it is reasonable to assume that the regular consumption of pulses is not a risk for children's growth. Concerning bioactive activity, data are promising but inconclusive. More studies are required, especially in human populations, to confirm the benefits.

#### **1.4 Pulses' recommendations and consumption in childhood**

There is no consensus on the amount of pulses children should consume. However, there are recommendations for children's consumption in countries' dietary guidelines that incorporate pulses in their food patterns. Marinangeli *et al.* (2017) summarized which countries recommend pulses in their food guides. Based on this work, we consulted the dietary guidelines of European countries and compiled the pulse dietary recommendations for children (Table 1.2) when this information was accessible.

Dietary recommendations for children over two years are incorporated into most national dietary guidelines. Under two years, recommendations for infants and young children are typically represented in separate guidelines. According to these recommendations, pulses should be introduced into infant feeding before the first year of life (Table 1.2). The transition from a dairy diet to a diet with complementary foods, usually between four and six months, is critical in forming eating habits. Babies have an innate fondness for sweet and salty tastes and an aversion to bitter tastes (Fewtrell *et al.*, 2017). Thus, introducing pulses is crucial for accepting these foods throughout life.

Countries like Greece, Portugal, and most recently, France (Gazan *et al.*, 2021) have pulses in a separate category from their national food guides, giving these foods greater prominence. Greece has recommended consumption three times a week and France twice a week, while in Portugal, daily consumption is recommended. Regarding serving sizes, the recommended amount varies according to age in Greece and Portugal. In France, quantities are not specified.

Spain has recommended consumption twice or three times a week but does not specify quantities. Ireland and the United Kingdom (UK) do not determine the consumption frequency. In Ireland, there is a recommendation that children's serving size should be half of the recommended for adults from the age of five. However, in the UK, quantities are not specified. There is significant variability in serving sizes and frequency of consumption recommended among countries.

**Table 1.2** Pulses dietary recommendations for children (until 13 years old) according to the dietary guidelines of different European countries

Country	Age of introduction	Frequency of consumption	Serving size (cooked legumes/pulses)	References
France	From 4-6 month	At least twice a week	<i>nsp</i>	<a href="https://www.mangerbouger.fr/manger-mieux/a-tout-age-et-a-chaque-etape-de-la-vie">https://www.mangerbouger.fr/manger-mieux/a-tout-age-et-a-chaque-etape-de-la-vie</a>
Greece	From 7 months	3 serving/week (on average)	40-150g (depending on age group)	<a href="#">diatrofikoiodigoi</a>
Ireland	From 6 months	<i>nsp</i>	Approximately 90g (half the adult serving that is ¾ cup)	<a href="http://www.gov.ie">gov.ie</a> - Healthy Ireland ( <a href="http://www.gov.ie">www.gov.ie</a> )
Portugal	From 8 months	From 1 to 3 years old: 1 serving/day  From 4 years old: 1 or 2 servings/day	80g	<a href="http://www.dgs.pt/promocao-da-saude/educacao-para-a-saude/areas-de-intervencao/alimentacao.aspx">www.dgs.pt/promocao-da-saude/educacao-para-a-saude/areas-de-intervencao/alimentacao.aspx</a>
Spain	<i>nsp</i>	2 or 3 times a week or more (recommendation for the general population)	<i>nsp</i>	<a href="http://www.observatoriodelainfancia.es/ficherosoia/documentos/Adolescentes">www.observatoriodelainfancia.es/ficherosoia/documentos/Adolescentes</a> ( <a href="http://thefamilywatch.org">thefamilywatch.org</a> )
United Kingdom	Between 9 and 12 months	<i>nsp</i> (Eat some pulses)	<i>nsp</i>	The Eatwell Guide - GOV.UK ( <a href="http://www.gov.uk">www.gov.uk</a> )

\**nsp*: non-specific.

Source: Links available on Food-based dietary guidelines (FAO, 2016) <http://www.fao.org/nutrition/education/food-dietary-guidelines/regions/countries/en/>.

Data on pulses consumption by children in Europe is very limited. However, this information is available in the National Food and Physical Activity Portuguese Survey in Portugal. It was a large study that created a national representative database. Among other details, it collected data about children's food consumption (3 months to 9 years) using a 2-day food diary. About 10% of the evaluated group were reported to eat legume grains for at least one day of the study, but the average consumption of cooked pulses was only 5g per day (Lopes *et al.*, 2017). It was overly lower than the Portuguese food guideline recommendation of 80g per day (Table 1.2).

Even though there is very little data available on pulses consumption by children, it is known that dietary patterns in European countries are characterized by low consumption of pulses (Szczebyło *et al.*, 2020), remaining below all the recommendations.

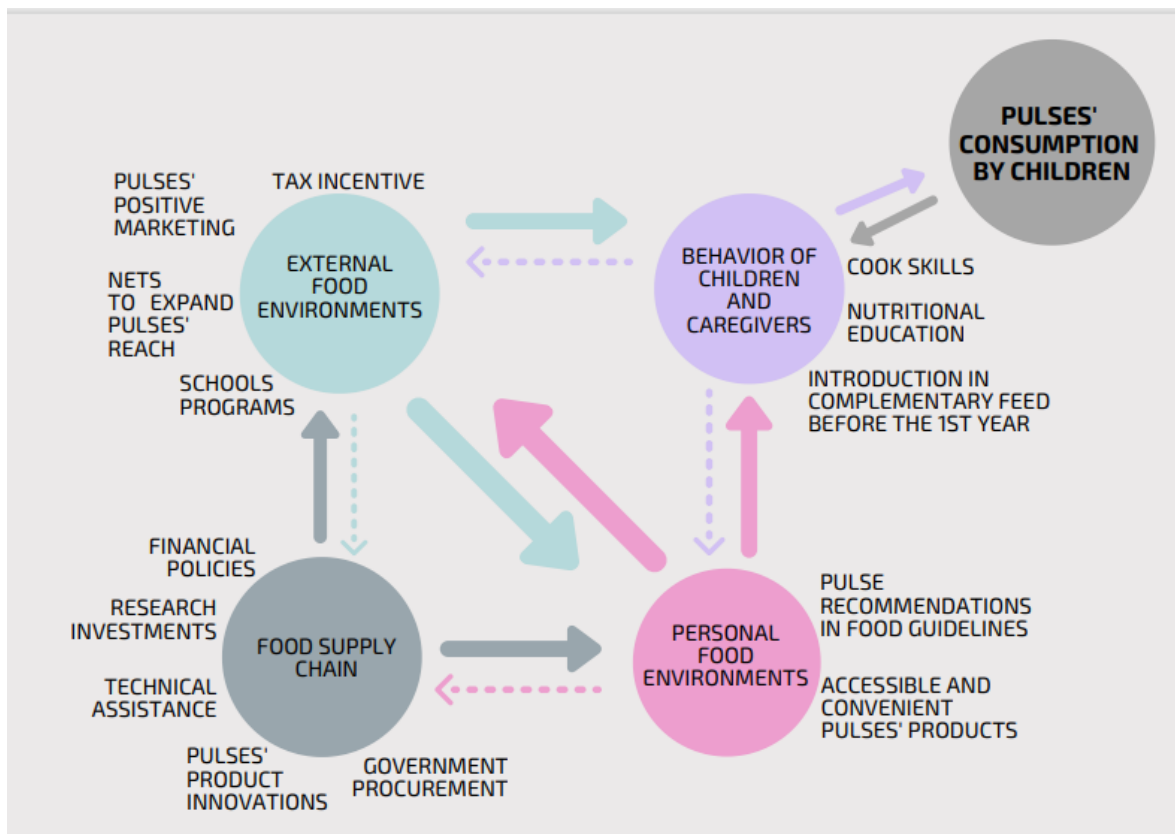
### **1.5 Promoting pulses' consumption in childhood**

Due to low consumption and environmental, nutritional, and health benefits, promoting pulses in children's diets is crucial for achieving more sustainable diets and food systems in Europe. Promoting pulses' consumption in childhood requires the governance of a set of actions with multiple complexity levels along with the food system. The *Innocenti* Framework, developed by the United Nations Children's Fund (UNICEF) and the Global Alliance for Improved Nutrition (GAIN), explains how the interaction among different actors in the food system interferes with children's and adolescents' diets (UNICEF and GAIN, 2019). It includes drivers (environmental, social, technological, demographic, political, and economic) and four determinants (food supply chains, external food environments, personal food environment, and behaviors of children and caregivers), which affect children's diets. In food policy development, it is essential to identify potential entry points for changes, focus on critical areas of intervention, and list the priority actions (Hawkes and Parsons, 2019; Parsons, 2019; Fanzo *et al.*, 2020). Here, the *Innocenti* Framework's structure was adopted to identify entry points for promoting pulses in children's diets and enable a systemic view of policy decisions.

This work development occurred in three stages. First, technical reports and articles from 2011 to 2021 were searched using the Food and Agriculture Organization (FAO) and the Medline databases. The review was conducted using the following terms: "Legume", "pulse", "sustainability", "environment", "food system", "nutrition", "children", and "policies". The title of the researched documents was analyzed, and all files that dealt with data referring only to specific countries or that did not have a theme directly related to the

review were excluded. After the first screening, the abstracts were read, and only documents that presented a clear path to health, nutrition, and sustainability, or addressed issues related to pulses' benefits, were included.

The second step selected the actions and measures (which included policies, programs, and campaigns). Actions that addressed the needs of the child population and were flexible enough to different age groups and socioeconomic conditions were prioritized. Finally, the measures were organized and discussed according to the *Innocenti* Framework determinants (Figure 1.3). In table 1.3, the actions that can support pulse consumption in childhood are compiled, and in the following sections, these actions are presented and discussed.



**Figure 1.3** Framework addressing solutions to promote pulse consumption by children  
\*adapted from the *Innocenti* framework (UNICEF and GAIN, 2019).

**Table 1.3** Policies that support pulses in children's diets

DETERMINANTS	ACTIONS	RESPONSIBILITIES	REFERENCES
Food supply chain	Financial policies	Governments	FSN Forum, 2016
	Research investments	Governments, academy	FSN Forum, 2016
	Technical assistance	Governments	FSN Forum, 2016
	Government procurement	Governments	Chakrabarti <i>et al.</i> , 2018
	Product innovations	Food industries, academies, governments	McDermott and Wyatt, 2017
External food environments	Nets to expanding pulses' reach	Governments, retailers	Rawal and Navarro, 2019
	School food environments	Governments, public and private schools	Beddington <i>et al.</i> , 2015
Personal food environment	Pulses' positive marketing	Governments, NGOs*, retailers, and food industries	McDermott and Wyatt, 2017
	Convenient pulses products	Food industries, academy	McDermott and Wyatt, 2017
	Recommendations in food guidelines	Governments	Hawkes, 2013
	Eco-labelling	Governments, retailers, food industry	HLPE Report, 2017
	Tax incentive	Governments	Beddington <i>et al.</i> , 2017
Behavior of children and caregivers	Nutritional education	Schools, health professionals, health centers, caregivers, nurseries, NGOs	Muehlhoff <i>et al.</i> , 2017; Ruel <i>et al.</i> , 2013
	Pulse cooking skills	Caregivers, schools, NGOs, health centers	Havemeier <i>et al.</i> , 2017; Fabbri and Crosby, 2016; Hawkes, 2013
	Introduce pulses in complementary diet before the first year	Caregivers, health professionals, nurseries	Grammatikaki, <i>et al.</i> , 2019

\* NGOs, non-governmental organizations

### *1.5.1 Actions in the food supply chain*

The food supply chain enrolls activities and players related to food production, storage, distribution, processing, retail, and markets. Food producers and suppliers choose food to grow, deliver, and sell, guided by several factors. Therefore, they choose which foods will be available in the food environments. Incentives and disincentives for certain foods' production directly impact the quantity, quality, and food price available (UNICEF and GAIN, 2019). Thus, interventions across food supply chains can improve pulses' availability, affordability, and acceptability.

For more sustainable diets, nutrition and environmental cost should guide the decisions within the agri-food systems, and the governments must align agriculture policies with nutrition policies (Eggersdorfer *et al.*, 2016). However, food production investments and research have prioritized staple crops and oilseeds over other crops with more considerable nutritional potential (HLPE Report, 2017). Compared to cereals, pulses have unstable yields and low returns, partly because they have been broadly forgotten in investments (Reckling *et al.*, 2016; Stagnari *et al.*, 2017). Policymakers should provide strategies to sustainably support pulses' integration into cropping systems, encouraging the production of locally adapted pulses. Suggested necessary actions that enable sufficient and consistent pulses supplies would be encouraging research in public and private sectors to develop high-yielding, abiotic/biotic stress-resistant varieties of pulses; support to smallholder producers by extension services and technical assistance, the supply of technologies and inputs; provide access to financing resources, and laws ensuring fair prices (FSN Forum, 2016). Supporting pulse production needs to be accompanied by measures to increase demand. Governmental procurement can align supply chains for sustainable pulses production. The procurement, followed by the distribution, has been an efficient initiative to stimulate the local food chain (Chakrabarti *et al.*, 2018).

### *Policies that promote pulses in value chains*

Experts considered that measures focusing on the pulse supply chain have a high potential to promote sustainable food systems if incentives for short food chains and domestic production are available (Balázs *et al.*, 2021). In addition, policy analysts claim that to observe a practical impact of pulses' production on sustainability, governments should determine goals and indicators for the long-term, provide legislation and infrastructure, promote intersectoral articulation, and encourage the creation of value chains

(Balázs *et al.*, 2019). Considering this, developing products using pulses from sustainable and local farming systems can potentially impact food sustainability. However, increasing pulses in value chains will not occur overnight. Governments must direct efforts to construct these value chains through policies and agreements enrolling farmers and the food industry, supported by the academy.

Most food ingredients used by the food industry are derived from wheat, corn, or soy. This usually results in products with a high degree of processing, poor nutritional profile, and rich in starches, sugar, and fat. Product reformulation reduces undesirable components, including minimally processed ingredients (Scrinis and Monteiro, 2017) to produce more nutritious, healthy, and sustainable foods is crucial. Pulses provide several opportunities to be utilized for this purpose, enhancing the nutritional quality and adding technological properties to foods (Clemente and Jimenez-Lopez, 2020). However, a limitation in adding pulses to food products is overall consumer acceptance (Binou *et al.*, 2020). Thus, pulse product innovations should mainly target children whose taste preferences are not yet established (Lartey *et al.*, 2016). Enriched foods with pulses can fortify these products naturally, improving micronutrient intakes by children (McDermott and Wyatt, 2017; Asif *et al.*, 2013). The children's nutritional needs should be central in creating such food products.

### *1.5.2 Actions in food environments*

Food environments are the food system interface related to food availability, accessibility, convenience, and desire (Downs *et al.*, 2020). The set of environmental, economic, social, and cultural characteristics converge to form the food environment in a community (Beddington *et al.*, 2017). These can be divided into external and personal food environments.

Food environments are crucial places to implement interventions for dietary changes because they contain the scope of options that can influence consumers' choices, with massive potential for impact (Downs *et al.*, 2020). Shaping food environments through measures and policies that determine food availability, price, and attractiveness can facilitate choices consistent with sustainable diets (FAO and WHO, 2019).

#### *External food environments*

The external food environments comprise the factors that interfere with food availability (UNICEF and GAIN, 2019). This dimension of food environments directly

relates to the food supply chain. Supply dynamics are directly responsible for increasing the availability of pulses in food environments. Studies that have examined how food availability shapes dietary intake have found a strong positive relationship between healthy food availability and its consumption (Caspi *et al.*, 2012). Therefore, it is fundamental to create structures that improve connectivity between rural, peri-urban, and urban supply and demand. It is also important to link small and medium pulse producers with food processors and retailers (including schools, kindergartens, and municipalities) or directly with the consumers. These are essential measures to expand the reach of pulse grains, pulse products, and pulse meals (Rawal and Navarro, 2019; HLPE Report, 2017).

In terms of shaping children's diets, the school food environment is a decisive space due to its strong influence on infant feeding. Schools have a highly favorable environment to promote and provide pulses for children and adolescents by making these foods available in school settings (Beddington *et al.*, 2017). In this sense, an excellent action to promote pulses' consumption among children is to make pulses more accessible and convenient in schools by incorporating pulses in school feeding programs, increasing pulses frequency on schools' menus, and offering pulse-based meals and snacks.

The school food environment is also an essential player in the food system due to its impact on other dimensions. For example, the food procurement for school meal programs can support local food supply chains and favor sustainability (Beddington *et al.*, 2015). The purchase of pulses from local farmers may strengthen sustainable agriculture in the region and create stable markets for producers (Beddington *et al.*, 2017). In addition, schools are the ideal place to carry out actions that can positively impact the behavior of children and caregivers, such as nutritional education and cooking skills. In the school food environment, nutrition education can go beyond the transmission of information and encompass strategies for behavior change (Hawkes, 2013). A systematic review of European studies found strong evidence that multi-component interventions in school food environments, coupling the rise of the availability of healthy foods with educational actions, can improve healthy food consumption among children (Van Cauwenberghe *et al.*, 2010). In this context, a set of strategies that combine information with increased exposure to pulses on the school menu could effectively increase their consumption in children's diets.

### *Personal food environment*

The personal food environment encompasses the factors that interfere with food choices at the individual and household levels, such as cultural factors, food cost and

affordability (economic access), convenience, desirability, and information (UNICEF and GAIN, 2019).

Food prices are an essential determinant of food demand. In recent years, overall pulse prices tended to rise, namely due to production's inability to increase at the same pace as the population (Bouis *et al.*, 2011). Measures already mentioned in the food supply chain to encourage pulses cultivation may reduce the prices. Also, fiscal measures, mainly through taxes and subsidies on food, may impact prices and influence consumers' purchasing and consumption choices. In high-income countries, subsidies for nutrient-rich foods tend to improve the diet's quality (Beddington *et al.*, 2017).

Food promotion is another factor that interferes with personal food choice. This type of strategy is usually underused by policymakers (FAO and WHO, 2019). Studies point out that food promotion actions strongly influence children's preferences, nutrition knowledge, and consumption patterns (Cairns *et al.*, 2013). A dedicated campaign developed by governments encouraging pulse consumption may positively impact dietary choices. Marketing concerted to an educational approach supported by policy from health, education, and environmental ministries could be highly effective. Also, disclosure strategies tailored to children and caregivers, such as branding, social marketing campaigns, and cooking contests, could be good initiatives developed by both the public and private sectors (McDermott and Wyatt, 2017).

A significant restriction for pulse consumption is their long preparation time (Szczebyło *et al.*, 2020), which indirectly lowers the availability of home-cooked pulses in children's diets. Innovations to reduce cooking time and produce more practical or "ready-to-eat" pulse-based foods could stimulate higher consumption (McDermott and Wyatt, 2017).

Regarding consumer information, a significant measure is to include pulse recommendations for children in national food guidelines. Dietary guidelines and nutritional recommendations provide information that influences individuals' food choices and orient food policies and industry decisions (Hawkes, 2013). Above, table 1.2 compiles pulses' recommendations for children in food guides from several countries. Another measure is to promote ecologically friendly labels that showcase the benefits of pulse products. According to European Union legislation, several nutrition claims should be applied for products that use pulses as an ingredient (Watson and Brandt, 2017). Besides shaping consumer preferences, nutrition labels can encourage the industry to reformulate the products (HLPE Report, 2017).

### 1.5.3 Actions related to the behavior of children and caregivers

Children's and caregivers' behavior is the factor that most directly influences children's diets. Parents and caregivers need to be oriented and supported to develop healthful home food environments and prepare healthy meals addressing children's food preferences (Fulkerson *et al.*, 2018). The younger the child, the higher the parents' and caregivers' responsibility for infant feeding, and the parents' knowledge, skills, experience, income, and time, all influence the food that is purchased, prepared, and provided (UNICEF and GAIN, 2019). Regarding pulses, low cooking and preparation skills continue to be a relevant barrier to their consumption (Palmer *et al.*, 2018). Literature suggests that developing pulse cooking skills and enhancing pulses' flavor through preparation may increase their consumption among children (Havemeier *et al.*, 2017; Fabbri and Crosby, 2016). Pulse cooking skills can be taught as a means of nutrition education for children and caregivers in schools and community kitchens (Hawkes, 2013). Also, the dissemination of pulse recipes and cooking books, especially when tailored to children's preferences, could help parents, caregivers, and schools to introduce pulse into children's diets. A recent example was developed by the H2020 project TRUE (TRUE Project, 2019).

Another efficient way to promote pulse consumption is to rescue the food cultural heritage of traditional populations. There is a richness of traditional pulse-based dishes that are often a staple to children around the world (FAO OCCP, 2016). These and other recipes adapted to contexts and modern family routines can be vehicles for promoting pulse consumption. One example of this type of initiative came from the Slow Food movement, which has been working to preserve the local culinary traditions across the globe. A cookbook with traditional and modern European pulse recipes was recently organized by Slow Food Deutschland (TRUE Project, 2018).

Personal preferences and tastes are also crucial for pulse acceptability. In many contexts, consumers reject pulses due to digestive intolerance, lack of habits, and taste rejection (Phillips *et al.*, 2015; Szczebyło *et al.*, 2020; Duarte *et al.*, 2020). Here, introducing healthy and diverse foods in the complementary feed may help form taste preferences and good eating habits later in life (Grammatikaki *et al.*, 2019). Thus, pulses should be introduced into infant feeding early, before the first year of life (Table 1.1). However, in some places, there is a belief that pulses are not suitable for young children (Szczebyło *et al.*, 2020). In these cases, it is important to emphasize that after domestic processing (e.g., soaking and cooking) to remove antinutritional compounds, pulses are an excellent

complementary food to help infants and young children meet their daily nutritional demands (Trehan *et al.*, 2015).

Finally, an extremely relevant action that can promote pulse consumption is education in food and nutrition for children and caregivers. Nutrition education must stimulate critical analysis of food choices and help to develop skills that favor a healthy lifestyle (Brazil, 2012). Besides providing information, it is necessary to communicate strategies (motivational), facilitate people's ability to act, and change the food environment. Under this definition, the ultimate goal of nutrition education is to change behavior (Hawkes, 2013).

Nutrition education is considered an essential component in improving children's food patterns, positively impacting children's nutrition status (Muehlhoff *et al.*, 2017; Ruel *et al.*, 2013). A nutritional education program to raise awareness about pulses' benefits, enrolling children, and caregivers, would be highly effective.

#### *1.5.4 Advancing through Feasible Measures.*

Managing food policies is a big challenge since it requires the articulation of actions at multiple levels and the involvement of multiple actors in and outside governments (Parsons, 2019). A set of policies can promote pulses in children's diets while enabling more sustainable food systems. The implementation of these policies demands coordination of actions and decisions about priorities. Considering the impact and feasibility of the identified actions, innovation of children's food products with pulses and actions in school food environments seem to be priority measures because they are relatively simple to operate and have a high impact potential. These two measures can enroll several food system interfaces if well-articulated within this system.

Driven by sustainability and nutrition advantages, the number of pulse-based innovative food products has risen substantially in the last decade (Binou *et al.*, 2020). As a result, it is possible to find a wide range of pulse products in European markets, especially flours, snacks, bread, cakes, cookies, and pasta (Ferreira *et al.*, 2021). However, except for the baby food segment, few pulses innovations are available for children. Developing pulse products targeting children may foment sustainable production while incrementing the product's nutrient profile. Thus, new children's pulse products should be validated in environmental and chemical terms.

Products' environmental impact has been demonstrated using the life cycle assessment (LCA) approach, which effectively evaluates the environmental impacts of

products, processes, or activities. LCA is a tool that quantifies the emissions of all inputs and outputs associated with the production processes, throughout the entire life cycle, from resource extraction to disposal (Üçtuğ *et al.*, 2019; Arvanitoyannis *et al.*, 2014). It is a particularly useful technique for comparing two or more alternative options in terms of environmental impacts or optimizing the design and production process. LCA has been standardized and follows these main steps: goal, scope, and boundaries; inventory analysis to identify material flows and energy; impact assessment; and interpretation to conclude the assessment (Mogensen *et al.*, 2009). Goals definition and scoping enroll setting general objectives for the LCA, describing boundaries of the system to be studied (cradle to grave, cradle to gate, gate to gate), and establishing the functional unit (Foster *et al.*, 2006). The functional unit should be related to the functions of the product (Üçtuğ, 2019). Inventory analysis compiles all required resources and all emissions to produce a functional unit in the system under investigation. Finally, the impact assessment process data is collected during the inventory analysis, allocating it qualitatively into impact categories. After that, the results are normalized (divided by per capita burdens) to allow comparisons (Arvanitoyannis *et al.*, 2014). Foods are among the products whose environmental performance can be evaluated by LCA (Arvanitoyannis *et al.*, 2014). Studies evidenced that pulses products presented, in general, a lower environmental cost while offering higher nutritional density when compared with similar products available in the market (Saget *et al.*, 2021; 2020). However, none of these included products specially elaborated to children's audience.

Metabolomics is the study of global metabolite changes in biological systems using technologies such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectrometry (Kim *et al.*, 2016). In recent years, metabolomics methods have appeared as very useful tools in the field of food science, with studies that include since crops metabolism, until food digestion and biotransformation, passing by food origin and authenticity, identification and quantifications chemical compounds, sensory science, food safety, and processing (Bayram and Gökırmaklı, 2018). Over the last few years, legumes' chemical composition has been analyzed by metabolomics technics, especially in terms of phytochemicals (Llorach *et al.*, 2019). Incorporating pulses in food matrices can produce compound alterations that influence food quality, and metabolomics is a promising tool for identifying such alterations. To our acknowledgment, there is no study treating this subject.

Besides food product innovation, actions in school food environments were the other measures identified to promote pulse consumption among children. Schools are a highly favorable place for healthy lifestyles, where most children spend a considerable time having

at least one big meal (Oliva Rodríguez *et al.*, 2013). The school food environment involves all spaces, infrastructure, and conditions inside and outside the school where food is made available, obtained, acquired, and consumed. The food environment shapes how accessible, affordable, desirable, and convenient specific foods are, depending on available information, promotion, and pricing. A healthy school eating environment encourages the community to make food choices that contribute to better nutrition (FAO, 2019). In this sense, these are essential means of enabling pulse in school environments.

Since 2017 in Portugal the vegetarian option has been mandatory on the menus of public canteens and cafeterias (Assembleia da República, 2017). Such attitudes increase the availability of pulses in schools, as they emerge as the main source of protein in these meals (Lima, 2018). However, few works have been presented Portuguese schools' strategies to promote pulse consumption.

## **1.6 Objectives, scope, and outline of the thesis**

This thesis's main objective is to develop and validate practical and viable solutions to promote pulses in the Portuguese agri-food system through children's diets, opening paths for creating local value chains that foster sustainability. Above, a review and discussion on the measures that should stimulate pulses in children's diets were presented. Considering the feasibility and impact, two of these actions were selected to be developed. The chosen actions were to enrich a child's product with a pulse and pulse promotion in the school environment. The specific objectives of the present work are: 1) Select the pulse more suitable to develop the product; 2) Develop a children's yogurt enriched with a pulse; 3) Make characterizations of the developed product; 4) Identify teachers' perceptions of consumption and acceptance of legume grains in selected Portuguese kindergartens; 5) Develop educational material in e-book format aimed at early childhood education teachers to guide them in promoting the consumption of legumes; 6) Evaluate the suitability and applicability of the developed material; 7) Discuss the potential of these measures in promoting pulses in the Portuguese agri-food system.

This thesis is organized into four major parts, subdivided into 6 chapters. Table 1.4 presents the thesis structure. An option was made to present the results in articles that were submitted or published in peer-reviewed journals. The current part (part I) comprises a general introduction, literature review, and thesis contextualization.

Part II of the thesis includes Chapters 2, 3, and 4, which discuss developing and characterizing a pulse-enriched yogurt target for children.

Broad bean and lupin are traditional pulses in the Mediterranean region (Martínez-Villaluenga *et al.* 2008), present in the Portuguese diet. Broad beans are among the pulses most efficient in nitrogen-fixing and one of the best crops in yield (Verni *et al.*, 2019; Caracuta *et al.*, 2015). Lupins are highly nutritious and have demonstrated health benefits (Ruiz *et al.*, 2019; Kouris-Blazos and Belski, 2016). Due to their agronomic and nutritional advantages, broad beans and lupins were the pulses chosen to be tested before the product development. Chapter 2 presents the results of an essay on the potential of broad bean (*Vicia faba*) and white lupin (*Lupinus albus*) flours to support the growth of probiotic bacteria, which may be associated with health benefits. Based on these results and preliminary sensory tests, lupin was the pulse chosen to be used in the developed product.

Over the past few years, several studies have focused on the fortification of foods with pulses or their flours, mainly centered on pastry products (Binou *et al.*, 2020). At current work, yogurt was the product chosen to be enriched with pulses, for being often consumed by young children (Moore *et al.*, 2018). Also, yogurt contributes to diet quality in childhood by providing adequate daily nutrient intake for many nutrients, particularly micronutrients (Tremblay and Panahi, 2017; Marette and Picard-Deland, 2014). Additionally, yogurt is a food appreciated by the Portuguese population. Research reported that 53.2% of Portuguese households consumed between 4 and 12 yogurt units per week (In Market, 2021).

Chapter 3 presents the development of yogurt enriched with lupin flour and its nutritional, rheological, and sensorial characterization. Also, the LCA approach is used to assess the product's environmental impact. The simplified LCA, used in the current study, applied LCA methodology using generic data (qualitative and quantitative) and standard modules for transport or energy production, focusing on the most important environmental aspects (European Environment Agency, 1997).

At the end of part II, chapter 4 displays the results of the metabolomic evaluation of the lupin-enriched yogurt by nuclear magnetic resonance (NMR) spectroscopy. In recent years, NMR spectrometry has been recognized as a powerful technique for discerning the chemical properties of complex mixtures, such as foods (Lu *et al.*, 2016), offering several advantages of being quantitative and highly reproducible (Ramakrishnan and Luthria, 2016). NMR spectroscopy can be particularly suitable for investigating fermentation dynamics due to its ability to provide an unbiased and inherently quantitative global chemical overview of complex samples (Trimigno *et al.*, 2020). Thus, the main metabolomics differences between yogurt enriched with lupine and control yogurt (natural) were highlighted.

Part III of the thesis deals with pulse promotion in the school environment. European children usually begin their school life in kindergarten, from 3 years old. Preschool age is an essential stage in developing educational programs aiming to form good food habits since this is a critical period when children start the socialization process and the formation of food preferences (Nekitsing *et al.*, 2018). The school environment will have a massive impact on the formation of food tastes and preferences of these children (Beddington *et al.*, 2015). Thus, the kindergartens were the settings chosen to develop actions to promote pulses. In practical activity planning, it is essential to support teachers and caregivers in implementing promotion actions. Accordingly, preschool teachers' guidance material on promoting pulse consumption was prepared. After that, a focus group was carried out to know teachers' perceptions of the consumption of pulses in selected Portuguese kindergartens and evaluate the material produced.

Chapter 5 brings the results of an *online* focus group with Portuguese kindergarten teachers on legume consumption and evaluating material produced. The material developed is available as a thesis appendix.

Finally, in the last part (Part IV), chapter 6 discusses the potential of the chosen measures to promote pulses in the Portuguese agri-food system and presents the final remarks.

**Table 1.4** Thesis structure

<b>PART I.</b> <b>Introduction</b>	Chapter 1. General Introduction
<b>PART II.</b> <b>Pulse enriched yogurt</b>	Chapter 2. Can broad bean ( <i>Vicia faba</i> ) and white lupin ( <i>Lupinus albus</i> ) flours act as carbon sources to support probiotic growth?
	Chapter 3. Nutritional, rheological, sensory characteristics and environmental impact of a yogurt-like dairy drink for children enriched with lupin flour
	Chapter 4. Metabolic evaluation of lupin-enriched yogurt by nuclear magnetic resonance metabolomics
<b>PART III.</b> <b>Pulses promotion in Portuguese kindergartens</b>	Chapter 5. Legumes in Portuguese kindergartens: Analysis of frequency and acceptance level, and evaluation of an educational e-book for legumes promotion
<b>PART IV.</b> <b>Discussion and final remarks</b>	Chapter 6. General Discussion, conclusion, and perspectives

**PART II.**  
**Pulse enriched yogurt**

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**Chapter 2. Can broad bean (*Vicia faba*) and white lupin (*Lupinus albus*) flours act as carbon sources to support probiotic growth?**

This chapter will present the results of an assay that tested the capacity of broad bean (*Vicia faba*) and white lupin (*Lupinus albus*) flours to support probiotic bacteria's growth. This study resulted in a short communication that was submitted for publication.

## ABSTRACT

There is a growing interest in investigating substrates that support food probiotic growth. Pulses are an excellent source of nutrients and bioactive compounds, especially non-digestible oligosaccharides from the  $\alpha$ -galactosides group, constituting probiotic growth factors. This research aimed to evaluate the potential of white lupin and broad bean flours to support the growth of seven *Lactobacilli* and *Bifidobacterium* probiotic strains. Different MRS media were prepared using the flours, whole or dehulled, as carbon source, added in different concentrations (20, 30, 40, and 60 g/L), and were inoculated with 2% (w/v) of each probiotic strain. Viable cell numbers and medium acidification were monitored throughout fermentation and compared against those obtained with negative (MRS without carbon source) and positive (MRS with 20 g/L glucose) controls. White lupin at a concentration of 60g/L represented a good carbon source for both *L. acidophilus* Ki and *L. casei* ssp *paracasei* L26 and at concentrations of 40 g/L and 60 g/L for *B. animalis* Bb12. The flour concentration was more critical to probiotic growth than the composition (hull vs. dehulled). Results show that white lupin is a potential ingredient for developing functional foods.

Keywords: Broad bean; Lupin; *Bifidobacterium*; *Lactobacillus*

## 2.1 Introduction

Probiotics are live microorganisms that confer health benefits to the host when consumed adequately (Ji *et al.*, 2023). Ample evidence has confirmed the role of probiotics in treating many chronic diseases, modulating the host immunity, protecting against infectious and non-infectious diseases, and producing several beneficial substances such as organic acids and short-chain fatty acids (Yadav *et al.*, 2022; Sharifi-Rad *et al.*, 2020).

Due to all the benefits, consuming foods enriched with probiotics is increasing. The most common probiotics used in foods and dietary supplements are bacteria from the ancient genus *Lactobacillus*, *Bifidobacterium*, and the yeast species *Saccharomyces boulardii* (Ji *et al.*, 2023). However, some conditions must be fulfilled to confer health benefits to the host. When used in foods, probiotics must remain viable and in proper concentrations after processing and during the product's shelf-life. Furthermore, they must survive the acidic pH conditions of the gastric environment, reach the small intestine, and colonize the gut host (Markowiak and Slizewska, 2017). Due to the difficulty of maintaining probiotic growth in some food matrices (Sharifi-Rad *et al.*, 2020), there is interest in investigating substrates that support these microorganisms' viability.

Pulses have emerged due to their health, nutritional, and environmental benefits. They are accessible, versatile, and functional ingredients, especially in the form of flours that allow their incorporation into various food matrices (Bresciani and Marti, 2019). Pulses are also rich in non-digestible oligosaccharides (NDO), named raffinose family oligosaccharides (RFOs), represented mainly by raffinose, stachyose, and verbascose (Cichońska and Ziarno, 2021). These are excellent growth factors for probiotic bacteria (Elango *et al.*, 2022).

Lupins (*Lupinus albus*) and broad beans (*Vicia faba*), two pulses traditionally present in the Mediterranean region, are rich in RFOs (Elango *et al.*, 2022). Recently, lupin use has been increasing due to its nutritional density and potential health benefits (Bryant *et al.*, 2022). Additionally, research has evidenced agronomical advantages in broad bean cultivation since it is one of the most efficient nitrogen-fixing and has the best legume crop yield (Verni *et al.*, 2019; Caracuta *et al.*, 2015). The global lupin market is projected to register a CAGR (Compound Annual Growth Rate) of 5,1% (Technavio, 2022). In contrast, the broad bean market is projected to register a CAGR of 4.0% (Mordor Intelligence, 2021) during the forecast period 2021-2026.

Only a few studies have demonstrated lupin and broad bean's capacity to support the growth of probiotic bacteria (Marinangeli *et al.*, 2020; Gullón *et al.*, 2015). Most of these

have been carried out using extracted fractions rather than whole grains, which are, in most cases, the natural form of consumption. Given the current rise in the interest in these pulses, it is essential to identify further uses. Therefore, this work's objective was to evaluate comparatively the effectiveness of white lupin and broad bean flours, whole or dehulled, at different concentrations, as a carbon source to support the growth of seven probiotic strains of *Lactobacilli* and *Bifidobacterium*. These findings could support the development of functional foods.

## 2.2 Materials and methods

### 2.2.1 Broad bean and lupin flours

Broad beans (*Vicia faba*) and white lupins (*Lupinus albus*) were obtained from a local producer in Mirandela, Portugal. Both grains were stored in plastic bags in a dark place at room temperature. Whole and dehulled grains were milled to a fine powder using a commercial food processor Thermomix® TM31 (Vorwerk, Germany).

### 2.2.2 Microorganisms

Seven commercial probiotic strains were used in this study: *Lactobacillus acidophilus* Ki and LAFTI® L10, *Lactobacillus casei* LAFTI® L26 (currently *Lacticaseibacillus casei*), *Lactobacillus rhamnosus* R11 (currently *Lacticaseibacillus rhamnosus*), *Bifidobacterium animalis* Bb12 and Bo, and *Bifidobacterium breve* NCIMB (National Collection of Industrial and Marine Bacteria) 702258. *Lactobacillus acidophilus* Ki and *B. animalis* Bo, previously isolated from fermented milk, were obtained from CSK (Netherlands); *B. animalis* Bb12 was obtained from Christian Hansen (Denmark); *L. acidophilus* LAFTI® L10 and *L. casei* LAFTI® L26 were obtained from DSM Food Specialties (Australia) and *L. rhamnosus* R11 was obtained from LALLEMAND Bio-Ingredients (Canada). All strains were provided as ultra-frozen concentrates.

The different strains were reactivated, pre-inoculated, prepared in de Man-Rogosa-Sharpe (MRS; Biokar Diagnostics, France) broth, and incubated overnight at 37 °C. At least two subsequent culturing steps using the same growth conditions were performed after the first inoculation of about 100-200 µL concentrate in 15 mL of MRS broth. Cell biomass was harvested by centrifuging (Sorvall LYNX 4000, Thermo Scientific) the final volume of 15 ml of MRS broth containing each strain at 5000 rpm for 20 minutes at 4°C and washed once with the same volume of 8.5 g L<sup>-1</sup> sodium chloride solution. After centrifugation, the supernatant was discarded, and the pelleted biomass was resuspended in 8.5 g L<sup>-1</sup> sodium

chloride solution. In the case of *Bifidobacterium animalis* Bb12 and Bo and *Bifidobacterium breve*, MRS broth was supplemented with filter-sterilized 0.5 g L<sup>-1</sup> of L-cysteine·HCl (Fluka, Switzerland) to lower the redox potential and incubated in a plastic anaerobic jar with a GasPak<sup>TM</sup>EZ sachet (Becton, Dickinson and Company, USA), to achieve anaerobic conditions.

### 2.2.3 Media

The basal medium used for the evaluation of probiotic growth-promoting properties of the lupin and broad bean flours was MRS broth prepared by the addition of the different ingredients to enable the conventional carbon source (glucose) substitution, i.e., 10 g L<sup>-1</sup> of peptone (Sigma-Aldrich, USA), 10 g L<sup>-1</sup> of meat extract (Merck, Germany), 5 g L<sup>-1</sup> of yeast extract (Biokar Diagnostics), 2 g L<sup>-1</sup> of di-potassium phosphate (Merck), 1.08 g L<sup>-1</sup> of tween 80 (Merck), 5 g L<sup>-1</sup> of sodium acetate (Merck), 2 g L<sup>-1</sup> of ammonium citrate tribasic (Sigma-Aldrich), 0.2 g L<sup>-1</sup> of magnesium sulfate (Merck), 0.05 g L<sup>-1</sup> of manganese sulfate (Sigma-Aldrich); in the case of the positive control the medium was added with 20 g L<sup>-1</sup> of glucose (Sigma-Aldrich). MRS without carbon source was combined with different concentrations (20, 30, 40, and 60 g L<sup>-1</sup>) of whole or dehulled lupin or broad bean flours. For *B. animalis* Bb12 and Bo, *B. breve*, and *L. acidophilus* Ki, all media were supplemented with filter-sterilized 0.5 g L<sup>-1</sup> of L-cysteine·HCl and incubated at 37 °C under anaerobic conditions as previously described.

### 2.2.4 Evaluation of pulse flours as a carbon source to support probiotic bacterial growth

The fermentability potential of lupin and broad bean flours was evaluated by screening the flour's incorporation, stirring, and sterilization methods (data not shown) to select the ones that would not affect the structure and activity of lupin and broad bean flours. Then, the impact of the pulses' hull on probiotic strain acidification and microbial growth was determined. For that, a test was conducted with whole-grain flour vs. dehulled-grain flour at three concentrations (20, 30, and 40 g L<sup>-1</sup>) and focusing on one single *Bifidobacterium* and one single *Lactobacillus* strain (i.e., *B. animalis* Bb12 and *L. acidophilus* L10, respectively). Upon testing those three concentrations, the scope range was broadened to 20, 40, and 60 g L<sup>-1</sup> of lupin and broad bean whole flours. These were tested in MRS medium with seven probiotic strains (*L. acidophilus* Ki, *L. acidophilus* L10, *L. rhamnosus* R11, *L. casei* L26, *B. animalis* Bo, *B. breve*, and *B. animalis* Bb12), to select the best combination pulse type/concentration based on microbial growth and acidification.

### *Incorporation of lupin and broad bean flour and sterilization method*

The flour was added to the basal medium without a carbon source at the intended concentration and then homogenized with an Ultraturrax (ICA Works, USA) for 3 minutes at 10.000 rpm to ensure optimum homogenization. The resulting medium was subsequently sterilized using an autoclave at 110°C for 10 minutes.

### *Determination of the dehulling effect on bacterial growth by viable cell determination*

MRS broth was prepared with glucose 20 g L<sup>-1</sup> and without any carbon source as positive and negative controls, respectively. To MRS broth without a carbon source, three different percentages of lupin and broad bean flours (20, 30, and 40 g L<sup>-1</sup>) were added as previously described. Each sterilized medium was transferred to previously sterilized 50-mL Schott® flasks (*L. acidophilus* L10) or 50-mL flat-bottom glass bottles with a narrow neck (*B. animalis* Bb12) and inoculated at 2% (v/v) with *L. acidophilus* L10 or *B. animalis* Bb12, respectively; less technological demanding strains were selected for this stage. The assay was performed in duplicate. The inoculated glass flasks were placed in an orbital incubator at 37°C with stirring at 150 rpm, and growth was monitored by enumerating viable cell numbers (CFU/mL) at 0, 4, 6, 8, and 24 hours (in duplicate). At each sampling time, decimal dilutions were prepared by using peptone-saline water (8.5 g L<sup>-1</sup> sodium chloride; 1 g L<sup>-1</sup> peptone) up to 10<sup>-6</sup> and plated on MRS agar using the Miles and Misra (1938) method.

### *Evaluation of probiotic growth-promoting potential by viable cell numbers determination and medium acidification*

To confirm the growth-promoting potential of the wholegrain pulse flours, seven probiotic strains were selected: *L. acidophilus* L10, *L. acidophilus* Ki, *L. casei* L26, *L. rhamnosus* R11, *B. animalis* Bb12, *B. animalis* Bo, and *B. breve*. Such an array of strains was used since it has been demonstrated that using a specific carbon source is variable among different genera, species, and even strain levels. Growth curves were monitored by enumeration of viable cell numbers, and medium acidification was measured by pH measurement.

MRS basal broth was prepared with glucose at 20 g L<sup>-1</sup> (positive control) or without a carbon source (negative control). To MRS basal broth without a carbon source, three different concentrations (20, 40, and 60 g L<sup>-1</sup>) of whole-grain pulse flours were added as previously described. In the case of the aerobic strains, *L. acidophilus* L10, *L. casei* L26, and

*L. rhamnosus* R11, 100-mL Schott® flasks were used for inoculation, while for anaerobes, *B. animalis* Bb12 and Bo, *B. breve* and *L. acidophilus* Ki, previously sterilized flat-bottom glass bottles with a narrow neck were used, to minimize oxygen contact. Each probiotic strain was inoculated at 2% (w/v) in each experimental medium, and the assay was performed in duplicate.

The glass bottles (anaerobic microorganisms) and Schott flasks (aerobic microorganisms) were incubated, as discussed above. Decimal dilutions and plating conditions were described in the previous section.

In addition to the growth curves, acid production was checked by pH measurements during incubation to confirm that bacteria used the substrates as a carbon source during the fermentation process. The pH meter was calibrated using reference pH 4.0 and 7.0 buffer solutions. The samples were blended before pH measurements.

#### 2.2.5 Statistical analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). The normality of the data was evaluated using Shapiro-Wilk's test. Data following normal distribution were analyzed using One-Way ANOVA coupled with Tukey post hoc test. The data that did not achieve normality was evaluated using Kruskal-Wallis test coupled with Dunn's post hoc test. The differences between means were considered significant for p-values below 0.05.

## 2.3 Results and discussion

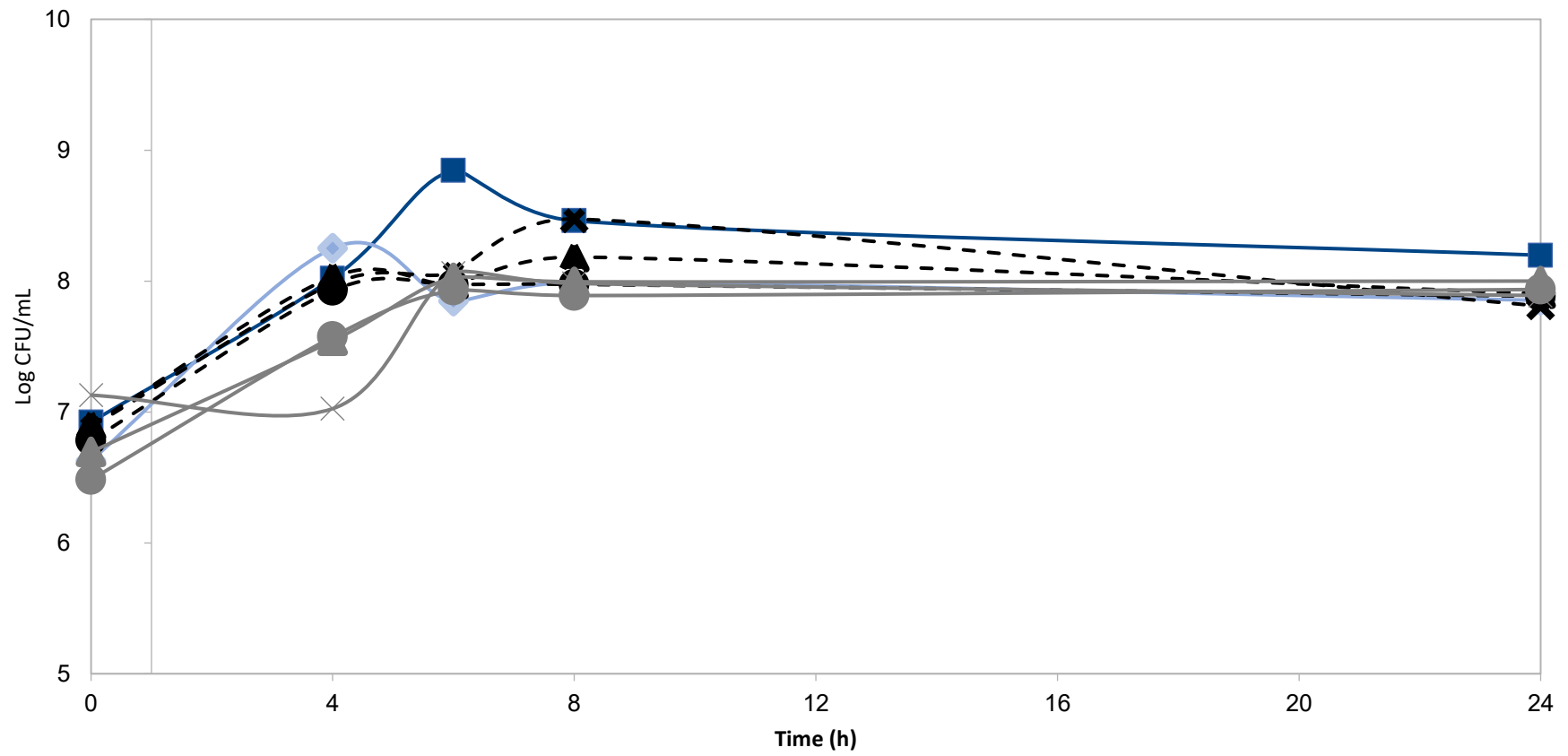
### 2.3.1 Determination of dehulling effect on bacterial growth

Growth in three different concentrations media (20, 30, and 40 g L<sup>-1</sup>) of pulse flour, over 24 h fermentation at 37 °C, were monitored by enumeration of viable cell numbers of *L. acidophilus* L10 and *B. animalis* Bb12 (Figure 2.1 a-d). Regarding carbon sources, glucose (positive control) stood out as the best choice for growth promotion (Figure 2.1) of both strains, in comparison to the alternative pulse flours – lupin and broad bean, independently of the presence or absence of a hull. There was no significant difference among groups (p>0.05). However, in the case of *B. animalis* Bb12 incubated in MRS basal medium containing 40 g L<sup>-1</sup> lupin flour, with or without the hull, viable cell numbers at 24 h fermentation were 1 log cycle higher than those reached in the MRS positive control. The results indicated that the absence of the hull did not have a greater impact on the growth behavior of *B. animalis* Bb12 when compared to the wholegrain counterparts. It can be

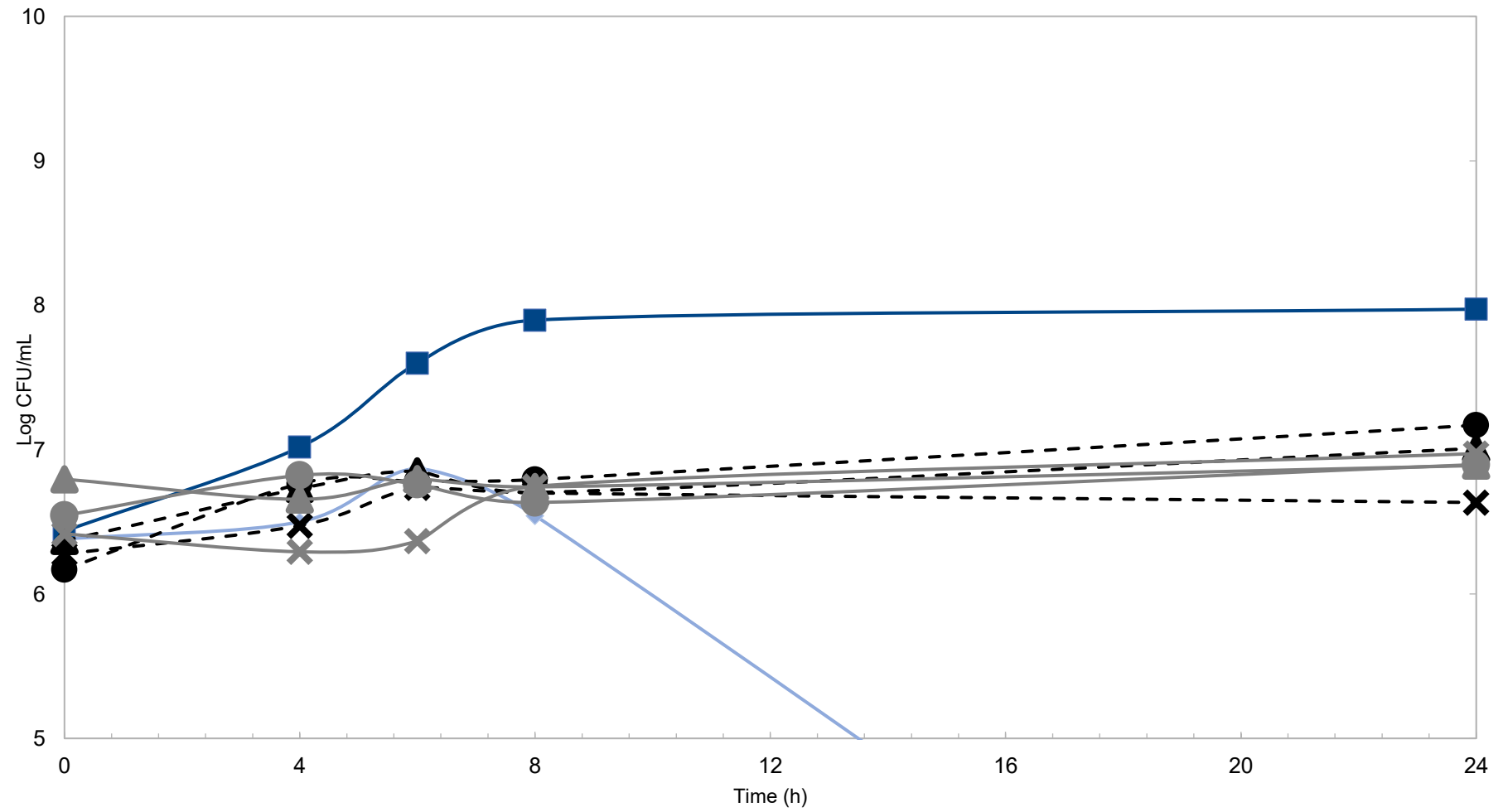
explained by the fact that the factors responsible for probiotic growth (mainly fiber and  $\alpha$ -galactosides) are contained within the seed's cotyledon (Cichońska and Ziarno, 2021).

In general, the dehulling of seeds reduces anti-nutrient compositions and leads to variations in nutrients (Choi *et al.*, 2023). In addition, research has shown that hull removal leads to lower antioxidant activity (Pal *et al.*, 2017), a biological property that should be maintained as high as possible. Thus, since there was no difference between using the lupin or broad bean flours, wholegrain or dehulled, it was more convenient to use the wholegrain flour for the following experiments because the preparation was easier, and nutrient losses were reduced.

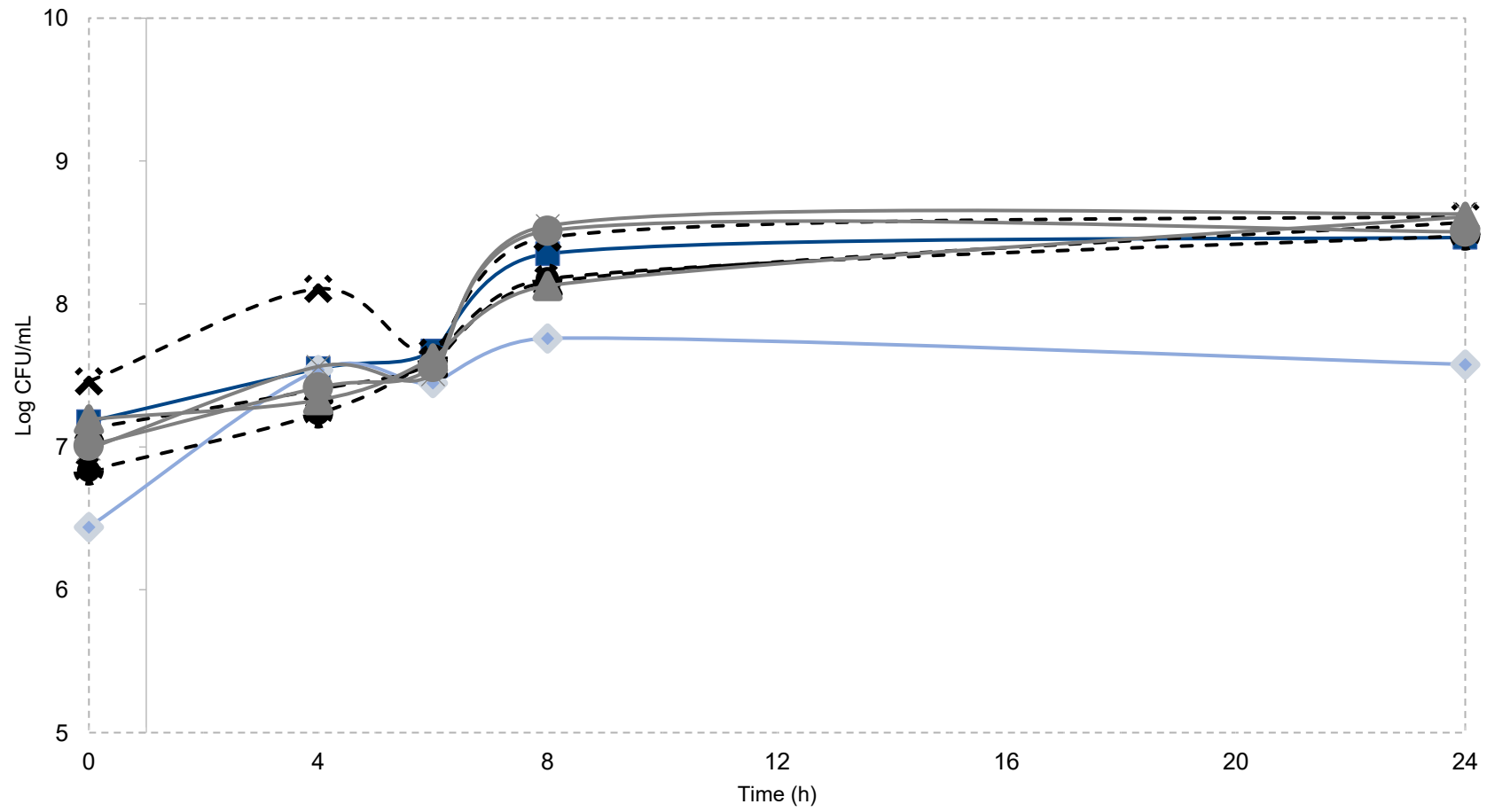
(a)



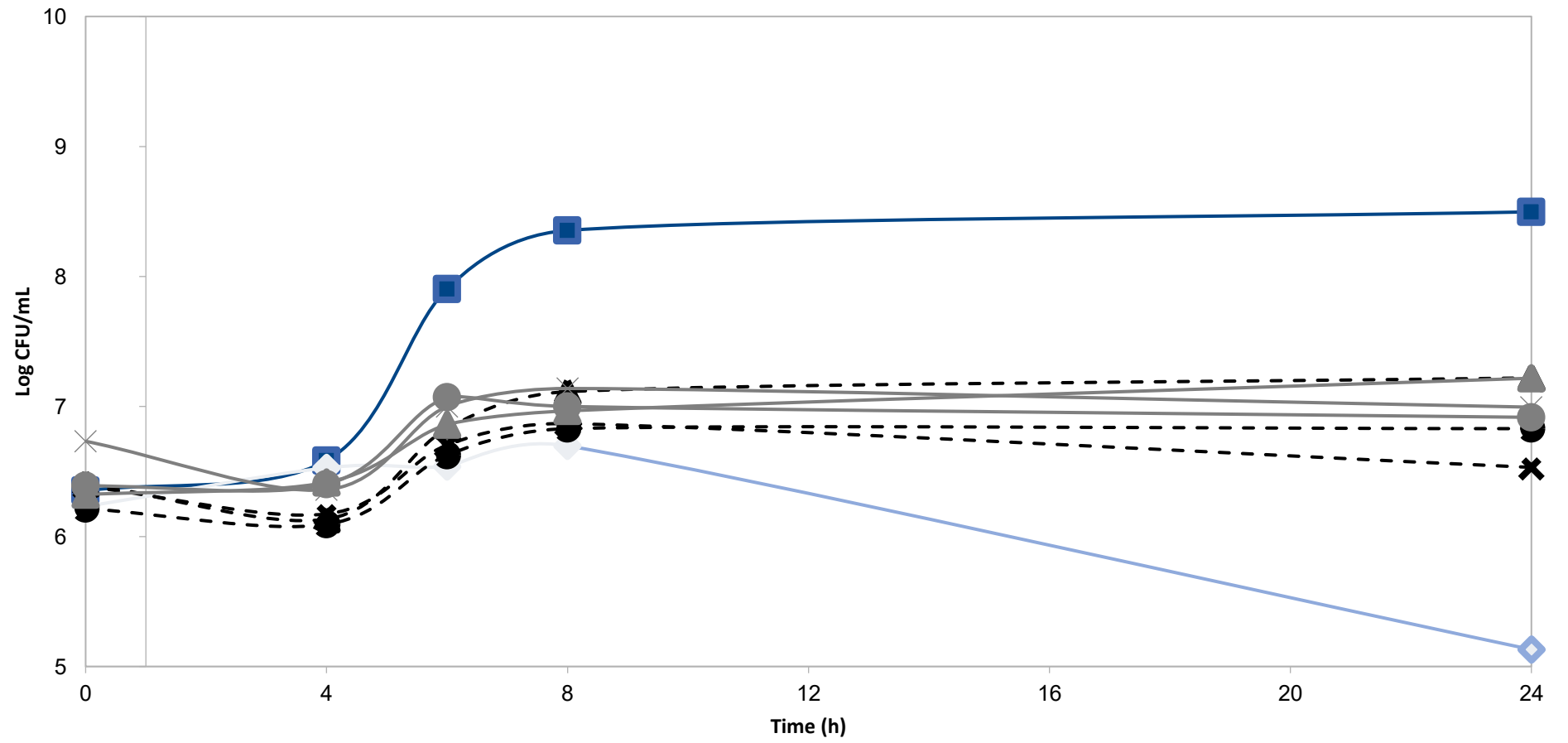
(b)



(c)



(d)



**Figure 2.1** Growth curves of *B. animalis* Bb12 (a and c) and *L. acidophilus* L10 (b and d) in MRS with (■) or without glucose (◆), and MRS with broad bean (a and b) or lupin (c and d) flour, with (—) or without (----) hull, at different concentrations: with 20 g L<sup>-1</sup> flour (▲), with 30 g L<sup>-1</sup> flour (●), with 40 g L<sup>-1</sup> flour (×)

### 2.3.2 Evaluation of probiotic growth-promoting potential of flours

To study the potential of pulses to promote the growth and acidification of probiotic strains, three concentrations of lupin and broad bean flours were added to the MRS basal medium without a carbon source and assessed on seven lactobacilli and *Bifidobacterium* strains. The growth curves are represented in Figure 2.2 (a-g), and the acidification capacity at the end of the 36-h fermentation period is shown in Table 2.1. In general, lupin flour supported the strains' growth more effectively than the broad bean flour at similar concentrations and, in some cases, produced similar or even better results than glucose.

The capacity of the different *Lactobacillus* and *Lacticaseibacillus* strains to utilize the legume flours as growth promoters reflected specificity at both species and strain levels, which agrees with former observations (Dong *et al.*, 2023; You *et al.*, 2022). The *L. acidophilus* Ki (Figure 2.2a) and *L. rhamnosus* R11 (Figure 2.2c) strains could metabolize the legume flours effectively added to the basal MRS media. At a concentration of 60 g L<sup>-1</sup> of lupin flour, bacterial growth of *L. acidophilus* Ki was significantly better ( $p < 0.05$ ) than that of the positive control from 12 h of fermentation (almost 1 log cycle higher). Also, the strain *L. rhamnosus* R11 registered a growth capacity similar to glucose by 36 h (9.3 log CFU/mL). However, the growth adaptation period was prolonged extensively to 12 h, which contrasted with that of 4 h to initiation of the growth logarithmic phase reported for glucose.

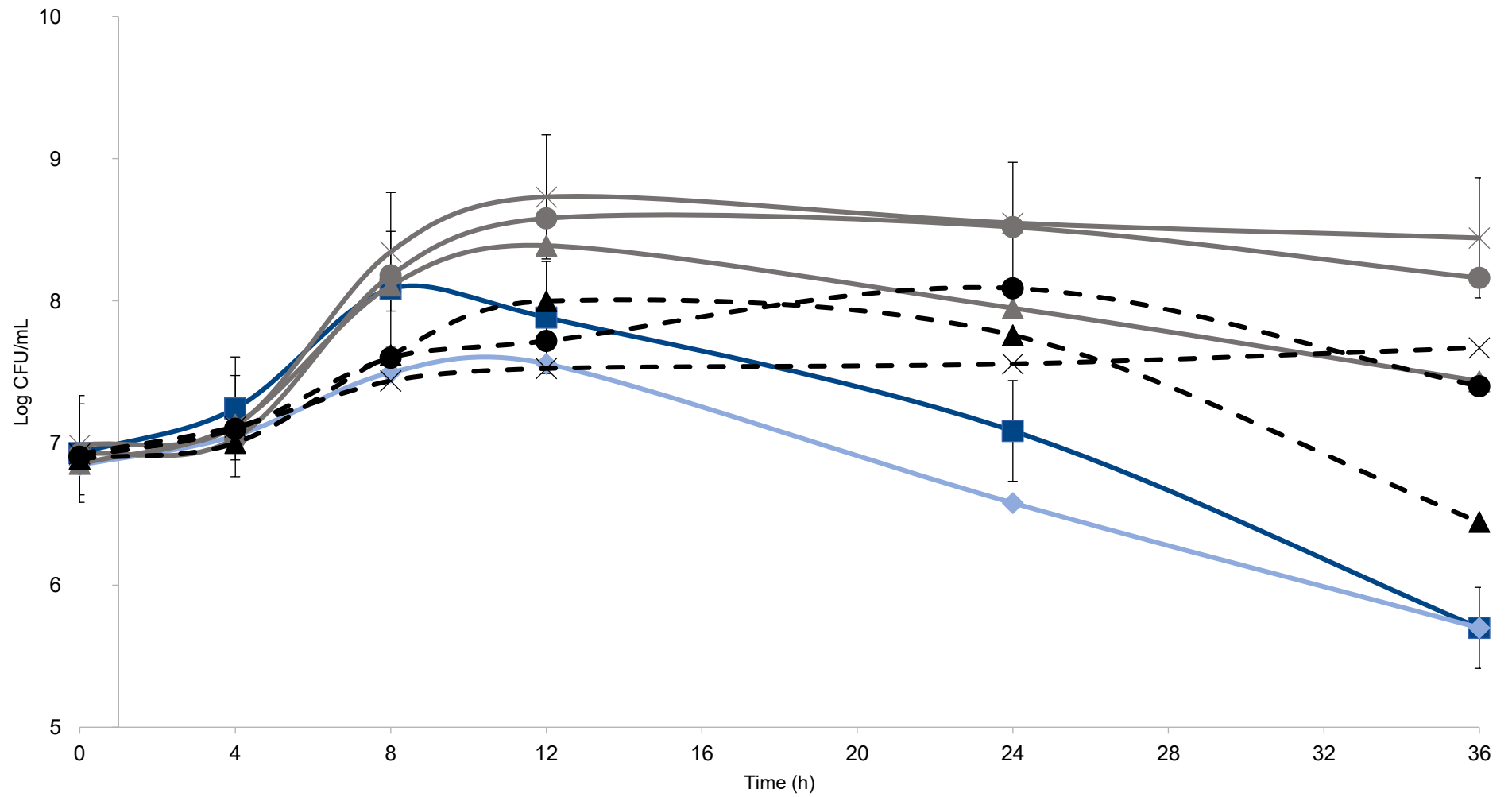
For *L. acidophilus* L10, both lupin and broad bean flours had no growth impact, independently of the concentration. Initial viable cell numbers were maintained relatively constant or decreased over time compared to those reported in the glucose medium (Figure 2.2b), supporting the strain-specific trait that may be observed for novel prebiotic sources. In the case of *L. casei* L26 (Figure 2.2d), viable cell numbers reached by 12 h (stationary phase) tended to be superior ( $p < 0.05$ ) with lupin flour (for all concentrations) and glucose compared to the broad bean flour addition.

All bifidobacteria strains showed a good capacity to use legume flours as carbon sources but to different extents. Higher concentrations of lupin and broad bean flours (40 g L<sup>-1</sup> and 60 g L<sup>-1</sup>) enabled *B. breve* to increment almost 3.0 log cycles, reaching similar viable

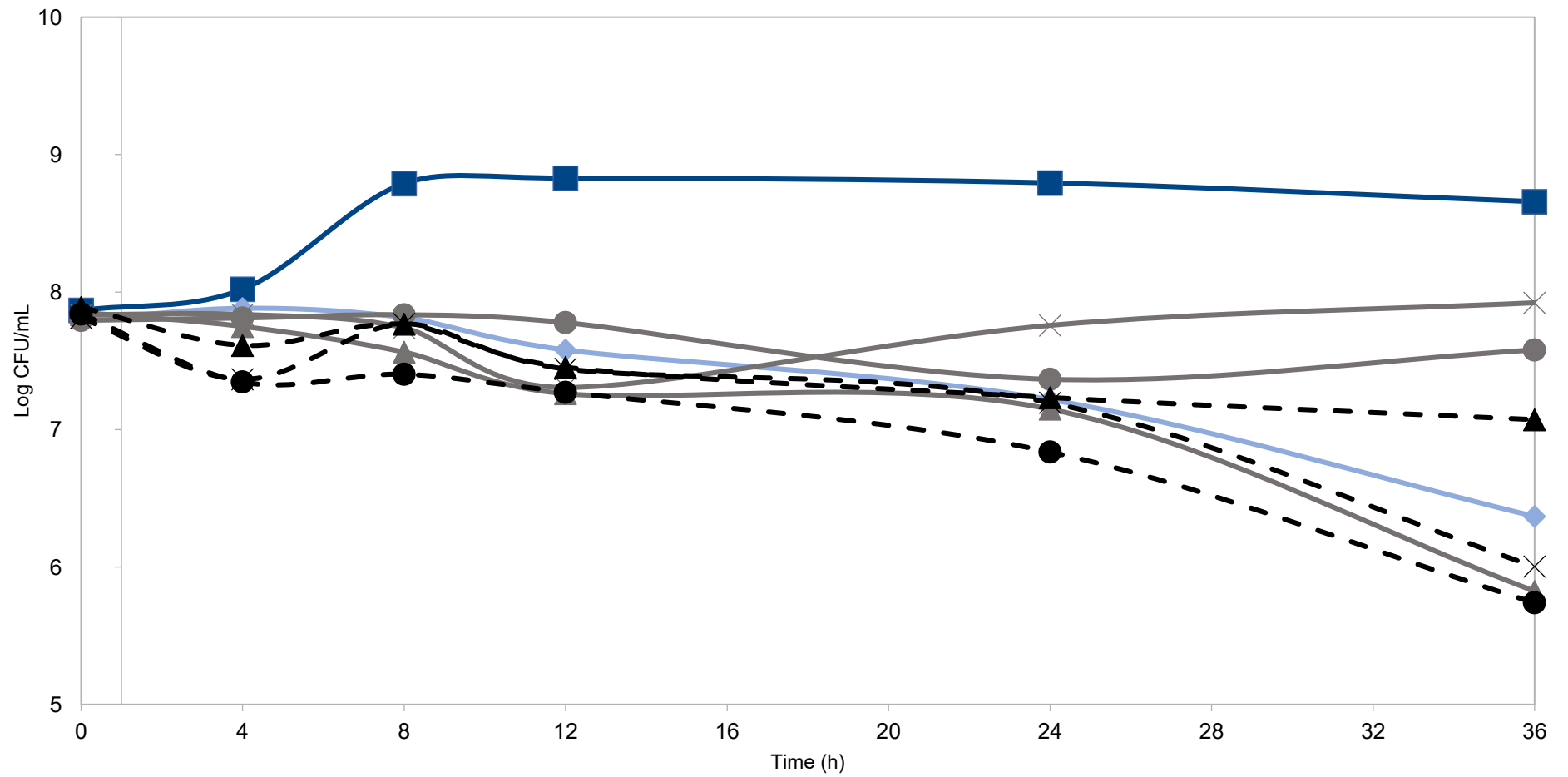
cell numbers to those reported for the positive control by 24 hours of fermentation (Figure 2.2f). At the same time, *B. animalis* strains (figures 2.2e and 2.2g) reported an increment of about 1.5 log cycles with concentrations of 40 g L<sup>-1</sup> and 60 g L<sup>-1</sup> of lupin flour.

For *B. animalis* Bb12, the growth curves of lupin flour at 40 g L<sup>-1</sup> and 60 g L<sup>-1</sup> were near the glucose curve (positive control), and, by 36 hours of fermentation, viable cell number (8.7 and 8.8 log CFU/mL, respectively) was significantly higher ( $p < 0.05$ ) than the other concentrations of lupin and broad bean flours (Figure 2.2g). In the case of *B. animalis* Bo, viable cell numbers in the presence of 60 g L<sup>-1</sup> of lupin flour were higher ( $p < 0.05$ ) than those of the positive control.

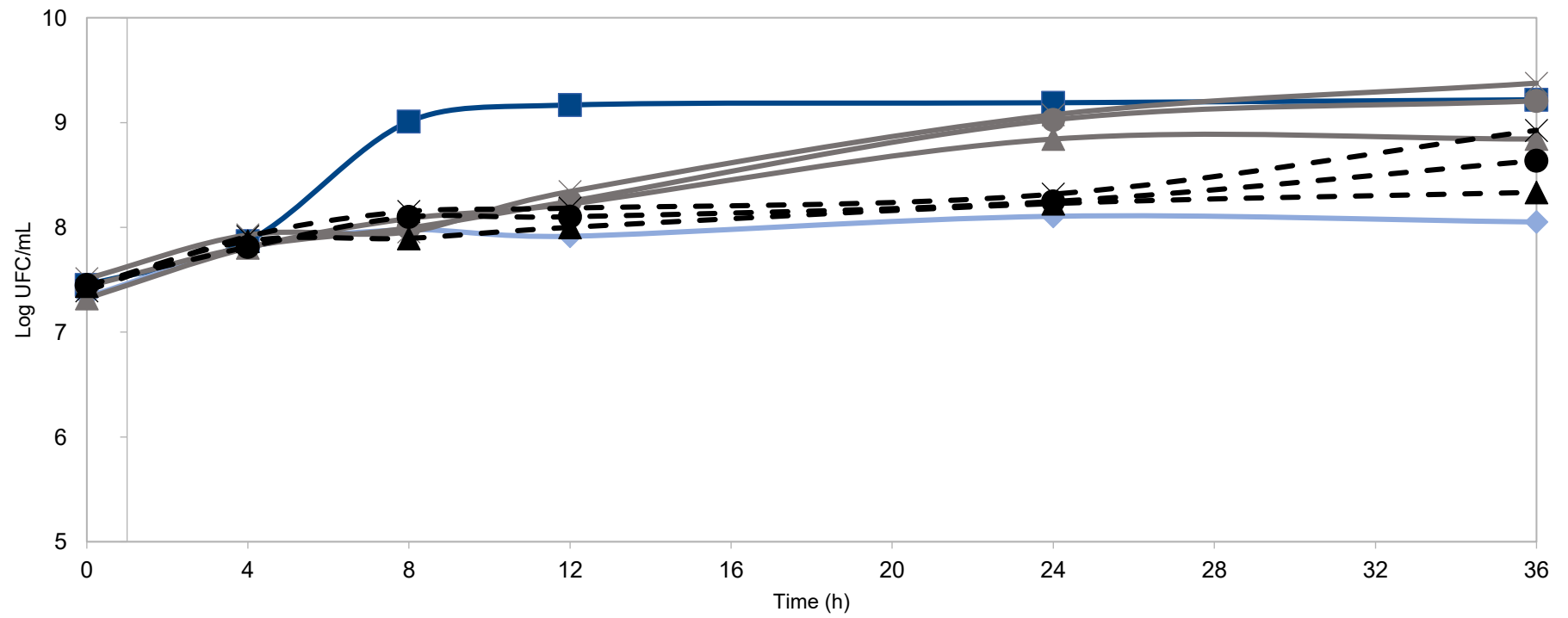
(a)



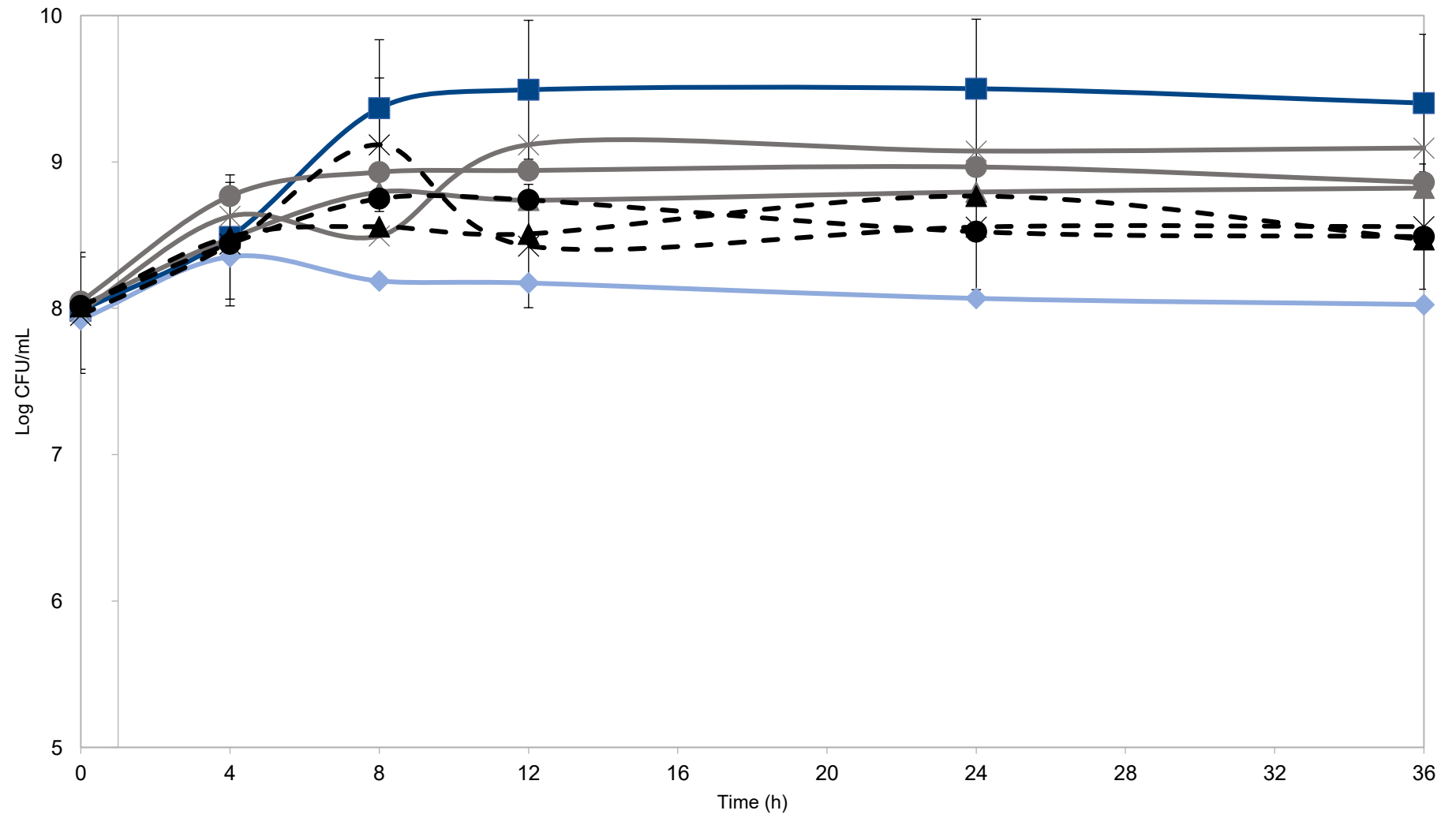
(b)



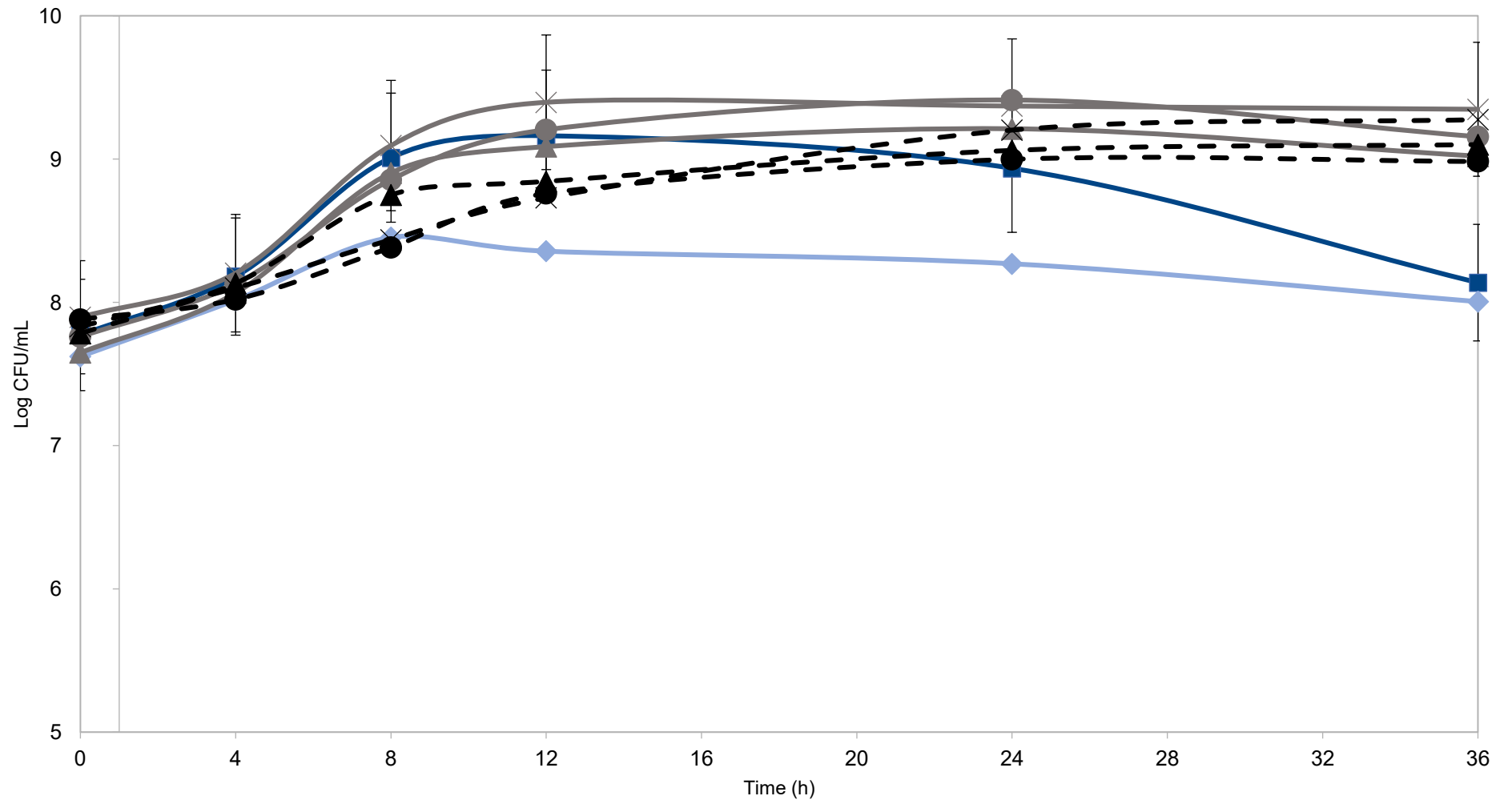
(c)



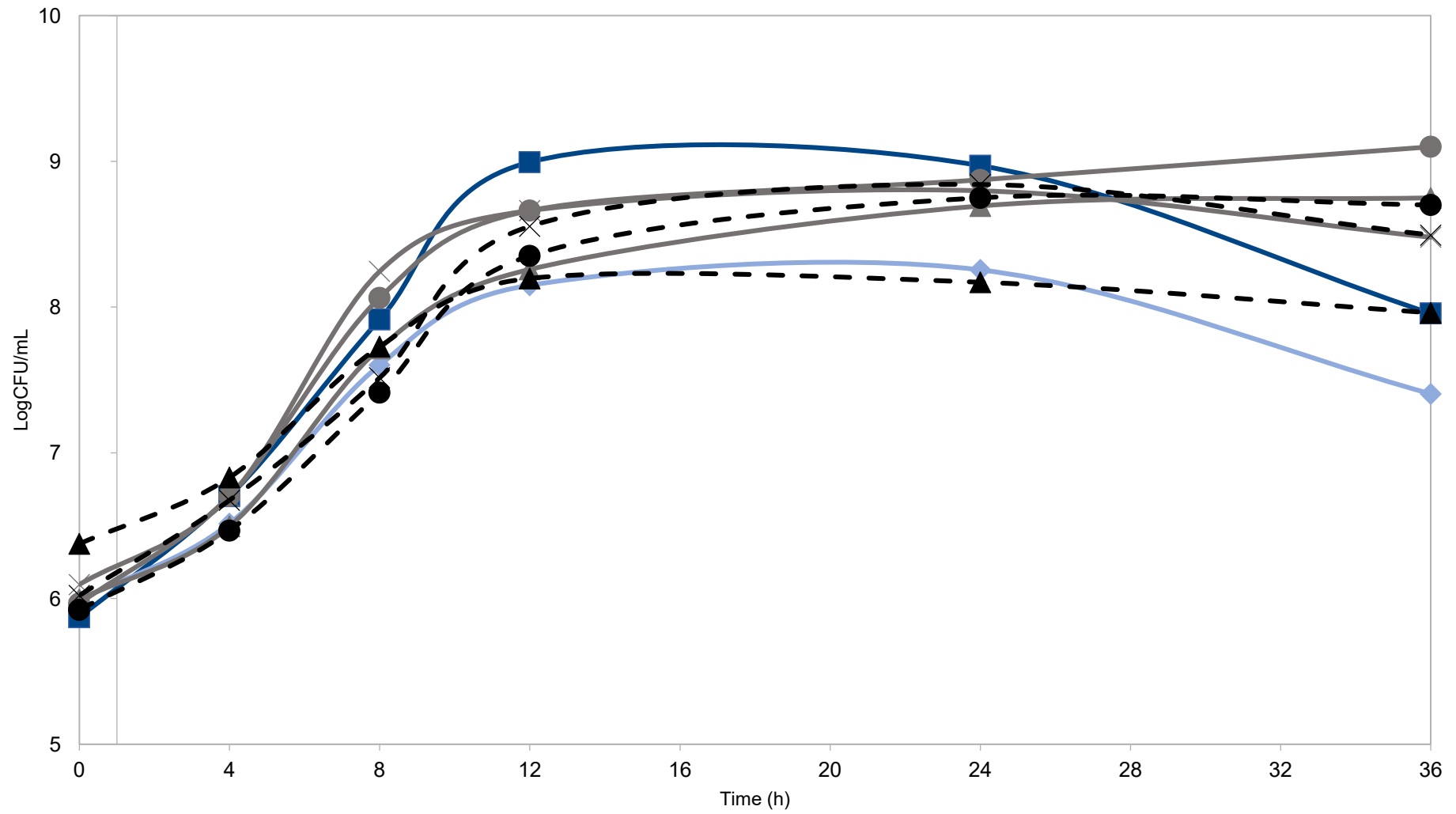
(d)



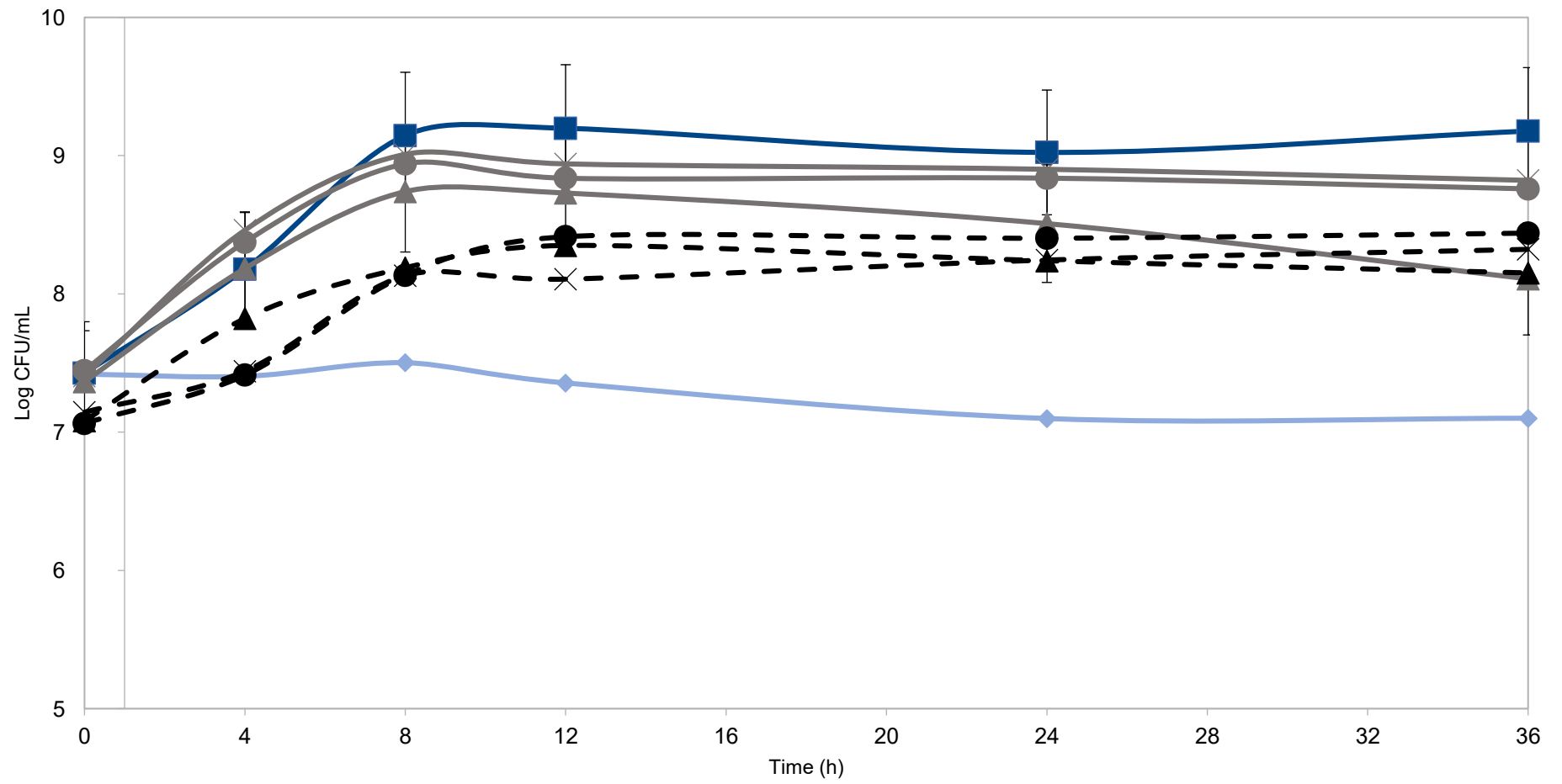
(e)



(f)



(g)



**Figure 2.2** Growth curves of *L. acidophilus* Ki (a), *L. acidophilus* L10 (b), *L. rhamnosus* R11 (c), *L. casei* ssp *paracasei* L26 (d), *B. animalis* Bo (e), *B. breve* (f), and *B. animalis* Bb12 (g) in MRS with (■) or without glucose (◆), and MRS with broad bean (----) or lupin flour (—), in different concentrations: with 20 g L<sup>-1</sup> flour (▲), with 40 g L<sup>-1</sup> flour (●), with 60 g L<sup>-1</sup> flour (×)

Metabolic activity is a good reflection of the growth capacity of strains, and in this study, it was followed by monitoring the evolution of acidification (pH decrease) throughout fermentation. In general, the growth curves of *Lactobacillus*, *Lactocaseibacillus*, and *Bifidobacterium* strains agree with the pH data presented (Table 1.1), indicating that these stains can effectively metabolize the legume flours, although at different rates and to different extents. As expected, glucose was the main carbon source (positive control), enabling the highest acidification rate independently of the species/strain being considered, and in the absence of a carbon source, acidification was insignificant. MRS basal media added with 40 g L<sup>-1</sup> and 60 g L<sup>-1</sup> of lupin flour and with 60 g L<sup>-1</sup> of broad bean flour tended to produce better results, similar to those reported for the positive control.

**Table 2.1** – Variation of pH for the seven probiotic strains tested in the different MRS culture media with or without glucose or supplemented with either 20, 40 or 60 g L<sup>-1</sup> of lupin or broad bean flours.

Strain	Incubation time (h)	Glucose		Lupin Flour			Broad Bean		
		20 g L <sup>-1</sup>	Without glucose	20 g L <sup>-1</sup>	40 g L <sup>-1</sup>	60 g L <sup>-1</sup>	20 g L <sup>-1</sup>	40 g L <sup>-1</sup>	60 g L <sup>-1</sup>
<i>L. casei</i> ssp <i>paracasei</i> L26	0	6.54±0.01 <sup>b</sup>	7.23±0.00 <sup>a</sup>	6.90±0.00 <sup>c</sup>	6.73±0.01 <sup>b</sup>	6.58±0.00 <sup>b</sup>	6.79±0.12 <sup>c</sup>	6.61±0.00 <sup>c</sup>	6.54±0.01 <sup>b</sup>
	4	5.68±0.04 <sup>b</sup>	6.8±0.02 <sup>a</sup>	6.39±0.01 <sup>c</sup>	6.25±0.00 <sup>b</sup>	6.14±0.00 <sup>b</sup>	6.17±0.01 <sup>c</sup>	6.11±0.07 <sup>c</sup>	6.11±0.01 <sup>b</sup>
	8	4.67±0.00 <sup>b</sup>	6.63±0.00 <sup>a</sup>	6.15±0.02 <sup>c</sup>	5.95±0.00 <sup>b</sup>	5.84±0.00 <sup>b</sup>	6.02±0.00 <sup>c</sup>	6±0.00 <sup>c</sup>	5.96±0.00 <sup>b</sup>
	12	4.21±0.00 <sup>b</sup>	6.66±0.02 <sup>a</sup>	5.95±0.02 <sup>c</sup>	5.67±0.00 <sup>b</sup>	5.5±0.02 <sup>b</sup>	5.91±0.00 <sup>c</sup>	5.9±0.02 <sup>c</sup>	5.81±0.00 <sup>b</sup>
	24	3.68±0.00 <sup>b</sup>	6.72±0.01 <sup>a</sup>	5.82±0.02 <sup>c</sup>	5.41±0.00 <sup>b</sup>	5.21±0.00 <sup>b</sup>	5.84±0.01 <sup>c</sup>	5.91±0.17 <sup>c</sup>	5.63±0.02 <sup>b</sup>
	36	3.81±0.00 <sup>b</sup>	6.79±0.02 <sup>a</sup>	5.84±0.04 <sup>c</sup>	5.43±0.01 <sup>b</sup>	5.25±0.02 <sup>b</sup>	5.81±0.01 <sup>c</sup>	5.86±0.18 <sup>c</sup>	5.56±0.08 <sup>b</sup>
	DpH	3.02	0.44	1.06	1.30	1.33	0.98	0.75	0.98
<i>L. acidophilus</i> Ki	0	6.49±0.00 <sup>b</sup>	6.78±0.00 <sup>a</sup>	6.55±0.00 <sup>ab</sup>	6.37±0.00 <sup>b</sup>	6.3±0.01 <sup>b</sup>	6.65±0.00 <sup>a</sup>	6.58±0.00 <sup>ab</sup>	6.52±0.00 <sup>ab</sup>
	4	6.23±0.00 <sup>b</sup>	6.66±0.00 <sup>a</sup>	6.36±0.00 <sup>ab</sup>	6.19±0.00 <sup>b</sup>	6.12±0.01 <sup>b</sup>	6.42±0.03 <sup>a</sup>	6.36±0.00 <sup>ab</sup>	6.29±0.00 <sup>ab</sup>
	8	4.81±0.00 <sup>b</sup>	6.26±0.00 <sup>a</sup>	5.54±0.04 <sup>ab</sup>	5.28±0.00 <sup>b</sup>	5.18±0.01 <sup>b</sup>	5.78±0.00 <sup>a</sup>	5.56±0.00 <sup>ab</sup>	5.45±0.00 <sup>ab</sup>
	12	4.32±0.00 <sup>b</sup>	6.26±0.00 <sup>a</sup>	5.15±0.04 <sup>ab</sup>	4.76±0.00 <sup>b</sup>	4.54±0.00 <sup>b</sup>	5.47±0.03 <sup>a</sup>	5.47±0.00 <sup>ab</sup>	5.33±0.00 <sup>ab</sup>
	24	4.01±0.04 <sup>b</sup>	6.21±0.00 <sup>a</sup>	5.12±0.03 <sup>ab</sup>	4.78±0.00 <sup>b</sup>	4.59±0.00 <sup>b</sup>	5.31±0.01 <sup>a</sup>	4.97±0.00 <sup>ab</sup>	5.2±0.00 <sup>ab</sup>
	36	3.96±0.04 <sup>b</sup>	6.24±0.00 <sup>a</sup>	5.14±0.03 <sup>ab</sup>	4.81±0.01 <sup>b</sup>	4.64±0.01 <sup>b</sup>	5.3±0.02 <sup>a</sup>	5.02±0.04 <sup>ab</sup>	4.84±0.01 <sup>ab</sup>
	DpH	2.53	0.54	1.41	1.56	1.66	1.35	1.53	1.68

<i>L. acidophilus</i> <b>L10</b>	0	6.72±0.00 <sup>b</sup>	7±0.12 <sup>a</sup>	6.82±0.00 <sup>a</sup>	6.62±0.01 <sup>b</sup>	6.47±0.00 <sup>b</sup>	6.93±0.01 <sup>a</sup>	6.83±0.00 <sup>a</sup>	6.73±0.04 <sup>c</sup>
	4	6.21±0.02 <sup>b</sup>	6.77±0.00 <sup>a</sup>	6.54±0.00 <sup>a</sup>	6.39±0.00 <sup>b</sup>	6.24±0.00 <sup>b</sup>	6.62±0.00 <sup>a</sup>	6.52±0.00 <sup>a</sup>	6.48±0.00 <sup>c</sup>
	8	4.76±0.01 <sup>b</sup>	6.97±0.00 <sup>a</sup>	6.79±0.02 <sup>a</sup>	6.64±0.02 <sup>b</sup>	6.52±0.01 <sup>b</sup>	6.81±0.00 <sup>a</sup>	6.85±0.02 <sup>a</sup>	6.84±0.02 <sup>c</sup>
	12	4.34±0.16 <sup>b</sup>	7.35±0.07 <sup>a</sup>	7.05±0.02 <sup>a</sup>	6.86±0.02 <sup>b</sup>	6.75±0.00 <sup>b</sup>	7±0.00 <sup>a</sup>	6.95±0.00 <sup>a</sup>	6.8±0.02 <sup>c</sup>
	24	3.77±0.00 <sup>b</sup>	7.19±0.01 <sup>a</sup>	6.94±0.00 <sup>a</sup>	6.8±0.00 <sup>b</sup>	6.65±0.00 <sup>b</sup>	7.01±0.01 <sup>a</sup>	6.84±0.02 <sup>a</sup>	6.81±0.00 <sup>c</sup>
	36	3.64±0.01 <sup>b</sup>	7.17±0.02 <sup>a</sup>	6.93±0.02 <sup>a</sup>	6.8±0.00 <sup>b</sup>	6.64±0.01 <sup>b</sup>	6.97±0.02 <sup>a</sup>	6.81±0.01 <sup>a</sup>	6.79±0.00 <sup>c</sup>
	DpH	3.08	-0.17	-0.11	-0.18	-0.17	-0.04	0.02	-0.06
<i>L. rhamnosus</i> <b>R11</b>	0	6.81±0.00 <sup>b</sup>	7.19±0.00 <sup>a</sup>	6.9±0.02 <sup>b</sup>	6.71±0.00 <sup>b</sup>	6.52±0.00 <sup>b</sup>	7.03±0.00 <sup>a</sup>	6.91±0.01 <sup>c</sup>	6.82±0.01 <sup>b</sup>
	4	6.13±0.00 <sup>b</sup>	6.72±0.00 <sup>a</sup>	6.46±0.00 <sup>b</sup>	6.33±0.00 <sup>b</sup>	6.18±0.00 <sup>b</sup>	6.59±0.01 <sup>a</sup>	6.48±0.00 <sup>c</sup>	6.42±0.00 <sup>b</sup>
	8	4.66±0.00 <sup>b</sup>	6.71±0.00 <sup>a</sup>	6.49±0.02 <sup>b</sup>	6.35±0.00 <sup>b</sup>	6.19±0.00 <sup>b</sup>	6.65±0.00 <sup>a</sup>	6.54±0.00 <sup>c</sup>	6.49±0.03 <sup>b</sup>
	12	4.21±0.00 <sup>b</sup>	6.75±0.02 <sup>a</sup>	6.58±0.01 <sup>b</sup>	6.38±0.00 <sup>b</sup>	6.19±0.00 <sup>b</sup>	6.66±0.01 <sup>a</sup>	6.58±0.00 <sup>c</sup>	6.5±0.02 <sup>b</sup>
	24	3.55±0.00 <sup>b</sup>	6.87±0.00 <sup>a</sup>	6.26±0.03 <sup>b</sup>	5.83±0.03 <sup>b</sup>	5.62±0.00 <sup>b</sup>	6.64±0.02 <sup>a</sup>	6.43±0.01 <sup>c</sup>	6.36±0.00 <sup>b</sup>
	36	3.57±0.00 <sup>b</sup>	6.95±0.00 <sup>a</sup>	6.08±0.00 <sup>b</sup>	5.58±0.03 <sup>b</sup>	5.33±0.02 <sup>b</sup>	6.59±0.00 <sup>a</sup>	6.45±0.00 <sup>c</sup>	6.31±0.01 <sup>b</sup>
	DpH	3.24	0.24	0.82	1.13	1.19	0.44	0.46	0.51
<i>B. animalis</i> <b>Bb12</b>	0	6.82±0.02 <sup>b</sup>	7.12±0.00 <sup>a</sup>	6.83±0.01 <sup>b</sup>	6.62±0.00 <sup>b</sup>	6.47±0.00 <sup>b</sup>	6.99±0.00 <sup>ab</sup>	6.86±0.00 <sup>b</sup>	6.77±0.00 <sup>b</sup>
	4	6.54±0.05 <sup>b</sup>	7.03±0.00 <sup>a</sup>	6.22±0.00 <sup>b</sup>	5.89±0.07 <sup>b</sup>	5.76±0.00 <sup>b</sup>	6.5±0.00 <sup>ab</sup>	6.46±0.00 <sup>b</sup>	6.38±0.00 <sup>b</sup>
	8	5.53±0.00 <sup>b</sup>	7.01±0.00 <sup>a</sup>	5.33±0.00 <sup>b</sup>	4.87±0.00 <sup>b</sup>	4.66±0.00 <sup>b</sup>	5.77±0.02 <sup>ab</sup>	5.35±0.04 <sup>b</sup>	5.13±0.02 <sup>b</sup>
	12	5.01±0.06 <sup>b</sup>	7.05±0.00 <sup>a</sup>	5.35±0.01 <sup>b</sup>	4.87±0.00 <sup>b</sup>	4.66±0.01 <sup>b</sup>	5.66±0.02 <sup>ab</sup>	5.17±0.02 <sup>b</sup>	4.93±0.01 <sup>b</sup>
	24	4.45±0.00 <sup>b</sup>	7.05±0.00 <sup>a</sup>	5.33±0.00 <sup>b</sup>	4.87±0.00 <sup>b</sup>	4.67±0.01 <sup>b</sup>	5.49±0.04 <sup>ab</sup>	5±0.01 <sup>b</sup>	4.76±0.01 <sup>b</sup>
	36	4.32±0.03 <sup>b</sup>	6.98±0.00 <sup>a</sup>	5.31±0.00 <sup>b</sup>	5.02±0.01 <sup>b</sup>	4.86±0.00 <sup>b</sup>	5.42±0.02 <sup>ab</sup>	4.95±0.01 <sup>b</sup>	4.68±0.01 <sup>b</sup>
	DpH	3.50	0.14	1.52	1.60	1.61	1.57	1.73	2.09
<i>B. animalis</i> <b>Bo</b>	0	6.6±0.01 <sup>b</sup>	6.9±0.00 <sup>a</sup>	6.6±0.01 <sup>a</sup>	6.5±0.00 <sup>b</sup>	6.3±0.00 <sup>b</sup>	6.8±0.00 <sup>a</sup>	6.8±0.04 <sup>a</sup>	6.6±0.00 <sup>ab</sup>
	4	5.8±0.02 <sup>b</sup>	6.4±0.00 <sup>a</sup>	6.2±0.00 <sup>a</sup>	6.0±0.00 <sup>b</sup>	5.9±0.00 <sup>b</sup>	6.3±0.00 <sup>a</sup>	6.2±0.00 <sup>a</sup>	6.2±0.00 <sup>ab</sup>
	8	4.3±0.00 <sup>b</sup>	6.1±0.04 <sup>a</sup>	5.7±0.06 <sup>a</sup>	5.5±0.04 <sup>b</sup>	5.4±0.00 <sup>b</sup>	6.1±0.04 <sup>a</sup>	6.0±0.00 <sup>a</sup>	6.0±0.00 <sup>ab</sup>
	12	3.9±0.01 <sup>b</sup>	6.1±0.01 <sup>a</sup>	5.5±0.07 <sup>a</sup>	5.3±0.05 <sup>b</sup>	5.1±0.00 <sup>b</sup>	6.1±0.08 <sup>a</sup>	5.9±0.00 <sup>a</sup>	5.9±0.02 <sup>ab</sup>
	24	3.6±0.02 <sup>b</sup>	6.2±0.11 <sup>a</sup>	5.5±0.06 <sup>a</sup>	5.3±0.06 <sup>b</sup>	5.1±0.00 <sup>b</sup>	5.6±0.02 <sup>a</sup>	5.4±0.03 <sup>a</sup>	5.4±0.02 <sup>ab</sup>
	36	3.6±0.00 <sup>b</sup>	6.1±0.11 <sup>a</sup>	5.6±0.07 <sup>a</sup>	5.3±0.06 <sup>b</sup>	5.1±0.00 <sup>b</sup>	5.7±0.13 <sup>a</sup>	5.3±0.00 <sup>a</sup>	5.2±0.00 <sup>ab</sup>
	DpH	3.00	0.80	1.00	1.20	1.20	1.10	1.50	1.40
<i>B. breve</i>	0	6.36±0.00 <sup>a</sup>	6.67±0.00 <sup>a</sup>	6.44±0.00 <sup>a</sup>	6.29±0.01 <sup>a</sup>	6.2±0.00 <sup>a</sup>	6.56±0.00 <sup>a</sup>	6.5±0.01 <sup>a</sup>	6.45±0.00 <sup>a</sup>
	4	6.35±0.01 <sup>a</sup>	6.66±0.00 <sup>a</sup>	6.44±0.00 <sup>a</sup>	6.28±0.00 <sup>a</sup>	6.19±0.00 <sup>a</sup>	6.55±0.00 <sup>a</sup>	6.49±0.01 <sup>a</sup>	6.43±0.00 <sup>a</sup>
	8	6.12±0.02 <sup>a</sup>	6.5±0.02 <sup>a</sup>	6.27±0.06 <sup>a</sup>	6.01±0.04 <sup>a</sup>	5.79±0.00 <sup>a</sup>	6.44±0.03 <sup>a</sup>	6.39±0.01 <sup>a</sup>	6.28±0.00 <sup>a</sup>
	12	5.02±0.02 <sup>a</sup>	6.14±0.03 <sup>a</sup>	5.75±0.04 <sup>a</sup>	5.4±0.06 <sup>a</sup>	5.08±0.02 <sup>a</sup>	6.06±0.02 <sup>a</sup>	5.88±0.02 <sup>a</sup>	5.63±0.00 <sup>a</sup>
	24	4.4±0.00 <sup>a</sup>	6.13±0.02 <sup>a</sup>	5.39±0.11 <sup>a</sup>	5.07±0.01 <sup>a</sup>	4.94±0.02 <sup>a</sup>	5.71±0.00 <sup>a</sup>	5.51±0.01 <sup>a</sup>	5.38±0.03 <sup>a</sup>
	36	4.35±0.02 <sup>a</sup>	5.68±0.08 <sup>a</sup>	5.24±0.06 <sup>a</sup>	5.01±0.03 <sup>a</sup>	4.89±0.02 <sup>a</sup>	5.46±0.06 <sup>a</sup>	5.4±0.00 <sup>a</sup>	5.31±0.03 <sup>a</sup>
	DpH	2.01	0.99	1.20	1.28	1.31	1.10	1.10	1.14

Values with the same superscript letters show no significant difference ( $p \geq 0.05$ ) in relation to the media at the same strain.

It was possible to observe that, in general, white lupin flour was a suitable carbon source to support the growth of both *Lactobacillus* and *Bifidobacterium* strains. Other studies (Gullón *et al.*, 2015; Fritsch *et al.*, 2015) have already observed that lupin flour is a suitable carbon source for probiotic bacteria. Gullón *et al.* (2015) found that both broad bean and white lupin stimulated the growth of probiotic bacteria such as *Bifidobacterium spp.*, *Lactobacillus – Enterococcus* group, among others. However, in the current study broad bean flour did not represent a significant carbon source for probiotic growth.

There are marked differences in the amount and the type of  $\alpha$ -galactosides present in lupin and broad bean. White lupins can have twice the  $\alpha$ -galactoside content compared to broad beans (Martínez-Villaluenga *et al.* 2008). Also, stachyose is the main  $\alpha$ -galactoside present in white lupin seed, while verbascose is usually undetected. On the other hand, in broad bean, stachyose content is the lowest among pulses, whereas verbascose is the main oligosaccharide present in this seed (Martínez-Villaluenga *et al.* 2008; Martínez-Villaluenga *et al.* 2005b). A study showed that probiotic bacteria ferment verbascose limitedly (Zartl *et al.* 2018), while some researchers have documented that lactic acid bacteria use stachyose and raffinose (Fritsch *et al.*, 2015). Therefore, those variations in oligosaccharide composition between white lupin and broad bean could explain the difference observed in the present study.

Studies carried out *in vitro* (Thambiraj *et al.* 2018), in food matrices (Martínez-Villaluenga *et al.* 2005a), *in vivo* (Palacio *et al.*, 2016), and in humans (Smith *et al.* 2006) have demonstrated that lupin, usually in the form of the fiber component extract, can promote probiotic bacterial growth. In our study, whole lupin flour presented similar results without any extraction process, suggesting that it may be possible to achieve lupin's prebiotic benefits with minimal processing.

## 2.4 Conclusion

The study revealed that white lupin flour, especially at concentrations of 40 g L<sup>-1</sup> and 60 g L<sup>-1</sup> (w/v), is an effective carbon source to support the growth of different *Lactobacillus* and *Bifidobacterium* strains, albeit the demonstrated specificity at both species and strain levels. This finding makes lupin flour a potential ingredient for producing functional food products.

### **Chapter 3. Nutritional, rheological, sensory characteristics and environmental impact of a yogurt-like dairy drink for children enriched with lupin flour**

From the results found in the essay carried out in Chapter 2 and the preliminary sensory tests, a children's yogurt enriched with 4% dehulled lupin flour was developed. It was characterized in nutritional, rheological, and sensory terms, and its environmental impact was assessed. This chapter is an original article published in the *International Journal of Gastronomy and Food Science*:

**Vieira EDF**, Styles D, Sousa S, Santos C, Gil AM, Gomes AM, Vasconcelos MW. 2022. Nutritional, rheological, sensory characteristics and environmental impact of a yogurt-like dairy drink for children enriched with lupin flour. *International Journal of Gastronomy and Food Science* 30: 100617.

## **ABSTRACT**

Studies have demonstrated that the addition of pulses to foods can make them more nutritious. We hypothesize that lupin flour adds nutritional benefits to yogurts. This study aimed to characterize a lupin-enriched yogurt in nutritional, rheological, and sensorial terms by a trained panel and assess its environmental impact using the life cycle assessment (LCA) approach. For comparison, natural yogurt and a commercial formula were used as controls. The developed yogurt is “high in protein” (7g/100g), “source of fiber” (1.9g/100g), and “source of omega 3” (53 mg/100g). The lupin yogurt was the stiffest with the highest viscosity than controls according to rheological parameters. There were no significant sensory differences between the lupin-enriched yogurt and the controls, although some undesirable sensory characteristics, such as bitterness, granularity, and after-taste, were observed. The environmental impact per 100 g serving was similar to natural yogurt and slightly worse regarding commercial yogurt but better when expressed per Nutritional Density Unit (NDU). Using lupin flour to enrich yogurts for children can be an alternative to producing more nutritious products.

**Keywords:** Lupin flour; Yogurt; Functional food; LCA.

### 3.1 Introduction

The functional food market is one of the fastest-growing segments in the food product development category as an increasing number of consumers are concerned about issues related to health (Paul *et al.*, 2020; Asioli *et al.*, 2017). Pulses have a high potential to be used in functional foods, as they are good stabilizers, sources of protein, dietary fiber, minerals, vitamins, polyunsaturated fatty acids (linoleic and linolenic), minerals, and phytochemicals, which are all important for human health (Foschia *et al.*, 2017). Considering the current European Union legislation, the use of pulses as an ingredient in foods may also allow some nutrition claims, especially regarding fiber and protein (Watson and Brandt, 2017). One way to classify food products' nutritional quality is using Nutri-Score, a nutrition label that is shown on the front pack and that establishes the nutritional characteristics using a five-letter code (A, B, C, D, and E) in different colors (Ferreiro *et al.*, 2021). Studies carried out in several European countries have highlighted that the Nutri-Score generally follows public health nutritional recommendations (Hercberg *et al.*, 2021).

Beyond favorable nutritional composition, pulses are economically accessible and benefit soil health (Boeck *et al.*, 2021). Investing in food innovations that diversify the use of pulses may promote their cultivation in sustainable cropping systems while improving products' nutritional quality (Bresciani and Marti, 2019). Several new pulse-based products have appeared in the market in recent years, especially innovations enriching cereal products with pulses (Bresciani and Marti, 2019; Magrini *et al.*, 2018). Nevertheless, overall consumer acceptance of products with pulses is still a challenge (Binou, *et al.*, 2020). Considering this, product innovations could prioritize incorporating pulses in animal foods (Magrini *et al.*, 2018) and preferentially target younger consumers whose taste preferences have not yet been established (Lartey *et al.*, 2016). According to dietary recommendations, pulses should be introduced into infant feeding before the first year of life. Due to their nutritional density, pulses' inclusion in children's diets can increment nutrient intake helping reach nutritional demands and may prevent overweight (Vieira *et al.*, 2021).

Market research suggests that parents are looking for innovative ways to incorporate vegetables and healthy proteins into their children's food patterns while contributing to environmental sustainability (Austen, 2018). Products' environmental impact is widely evaluated by the Life Cycle Assessment (LCA) approach, which consists of quantifying resource use and environmental emissions of all inputs and outputs

associated with the production process, from extraction to disposal (Dai *et al.*, 2020). The LCA processes have been standardized by ISO 14040 (2006) and ISO 14044 (2006) norms and have been shown to be a useful tool to evaluate the environmental impact of foods and diet patterns (Heller *et al.*, 2013). Recent LCA studies evidenced that pulse-based products present a lower environmental impact while offering higher nutritional density than similar products available in the market (Saget *et al.*, 2020; 2021). Thus, developing new children's foods enriched with pulses seems to be the right direction to take.

The dairy industry has invested in making its foods more nutritious and functional beyond conventional milk products (Paul *et al.*, 2020). Yogurts are popular fermented foods produced by fermentation of milk by dairy cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Evidence is accumulating that yogurt consumption as part of a healthy diet is related to healthier metabolic profiles in both children and adults (Moore *et al.*, 2018). Often present in the infant diet, yogurts can contribute significantly to babies' and children's daily nutrient intake, providing substantial amounts of protein and essential micronutrients such as calcium, potassium, zinc, phosphorus, and magnesium (Marette and Picard-Deland, 2014). The addition of pulses in yogurts can increment their nutritional profile with nutrients that are missing in the conventional formulations such as fibers and polyunsaturated fatty acids. Lupin (*Lupinus albus*), a pulse crop that grows in the Mediterranean region, is highly nutritive, has a low number of anti-nutrients, and provides beneficial functional, antioxidant, and prebiotic properties (Porres *et al.*, 2006; Gullón *et al.*, 2015). Furthermore, lupin is rich in minerals such as iron, zinc, and manganese (Villarino *et al.*, 2016), which are particularly important for children's cognitive and motor development (Beal *et al.*, 2021). We hypothesize that lupin flour adds nutritional benefits to yogurts. The aim of this study was to develop an enriched lupin yogurt, characterizing it in nutritional, rheological, and sensorial terms, and assessing its environmental impact using the LCA approach.

## **3.2 Materials and methods**

### *3.2.1 Yogurt development*

The yogurt was prepared as described in Figure 3.1. Organic semi-skimmed milk (Milhafre®, Azores, Portugal) was mixed with 6% (6.8 g/100 ml) of skimmed milk powder (Molico®, Nestlé Portugal) and enriched with 4% (4.16 g/100 ml) of de-hulled organic lupin flour originated from the European Union. The nutritional composition of

lupin flour is available in table 3.1. The mixture of milk and lupin flour was homogenized and heated to 90°C for 3.5 minutes. After cooling to 40°C, the preparation was inoculated with an organic powder preparation for yogurt (Natali®, France) containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. After inoculation, the milk enriched with lupin flour was fermented in a water bath at 42°C for approximately 6 hours (until pH reached 4.6-4.5). After fermentation, the yogurt was gradually cooled and stored at 4°C for 24 hours, until the analyses were carried out or frozen in case of nutritional analyses.

**Table 3.1** Nutritional information of lupin flour

Energy/Nutrient	In 100 g of the product
Energy (kcal)	358
Total fat (g)	12
Saturated fat acids (g)	1.5
Carbohydrates (g)	9.0
Fiber (g)	27
Protein (g)	40

Source: Product's label

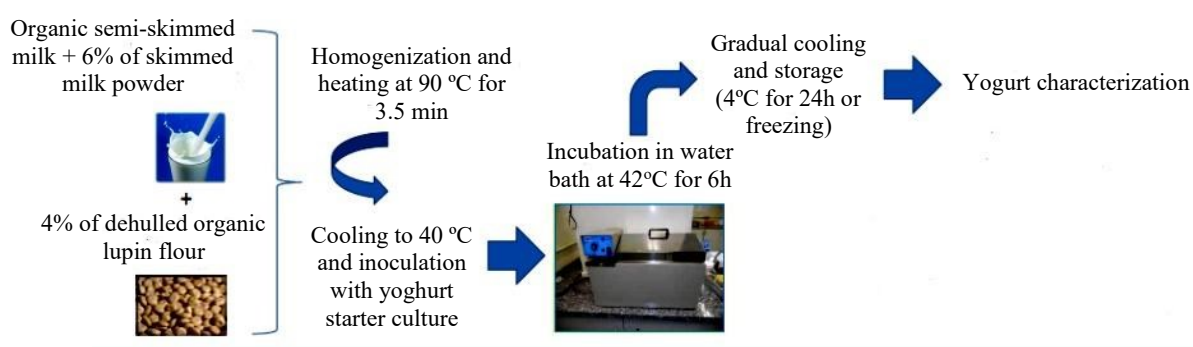
Two controls were used in the current study: i) a natural yogurt (NY), in which the preparation followed the same steps as the lupin enriched yogurt (LEY), except for the addition of lupin flour, and a commercially available infant yogurt formula (CY) (Table 3.2). The differences between the yogurts and commercial formula may limit some comparisons, but this was chosen because it was the product targeted to infants that was closer to yogurts. The three products were characterized concerning nutritional, physical, and sensory aspects.

**Table 3.2** The three yogurt formulations used in the current study and their ingredient lists.

Yogurt type	Ingredients
Lupin-enriched yogurt (LEY)	Organic semi-skimmed milk (90%), skimmed milk powder (6%), organic dehulled lupin flour (4%), and organic yogurt starter bacteria.

<b>Natural yogurt (NY)</b>	<b>Organic semi-skimmed milk (94%), skimmed milk powder (6%), organic yogurt starter bacteria.</b>
<b>Commercial yogurt (CY)</b>	Fermented milk (88.1%) [ <b>organic milk (60%*)</b> , water (24.1%*), organic <b>skimmed milk powder (3%*)</b> , thickener (pectin) (1%*), <b>yogurt starter bacteria</b> ], water (3.6%*), corn starch (2.3%*), rice starch (2%*), organic butterfat (3%*), organic rice semolina (1%*)

\* Estimated percentages.



**Figure 3.1** General workflow followed for the lupin-enriched yogurt production.

### 3.2.2 Yogurt characterization

#### *Nutritional Characterization*

The nutritional composition was determined in terms of macro-nutrients and minerals. The total protein content of the yogurt samples was obtained by the determination of total nitrogen by Kjeldahl method, with a nitrogen-to-protein conversion factor of 6.25 (AOAC, 1995). Moisture was determined in a vacuum oven using AOAC 925.09, and ash content was determined as a percentage after muffle incineration at 550 °C for 5 h (AOAC, 1995). Total fat was determined by the Gerber Method (Badertscher *et al.*, 2007), in which the separated fat is measured directly in a calibrated butyrometer. Fatty acids were quantified by gas chromatography coupled to a flame ionization detector (GC-FID) based on ISO 12966–1:2014; 12966–2:2011; 12966–3:2016 (ISO, 2016, 2014, 2011). The carbohydrate content was determined by differences based on protein, fat, moisture, and ash percentages. The conversion values for the macro-nutrients in Regulation (EU) No. 1169/2011 were considered for calculating the energy value. The

gravimetric enzymatic methods (AOAC 991.43 and AOAC 985.29) were applied to determine the total dietary fiber (AOAC, 2017).

Mineral analyses for iron (Fe), zinc (Zn), magnesium (Mg), manganese (Mn), copper (Cu), calcium (Ca), phosphorus (P), sodium (Na), and potassium (K) were performed using 200 mg of sample, analyzed in triplicate, mixed with 6 mL of 65% HNO<sub>3</sub> and 1 mL of 30% H<sub>2</sub>O<sub>2</sub> in a Teflon reaction vessel heated in a Speedwave<sup>TM</sup> MWS-3+ (Berghof, Germany) microwave system. Digestion procedures were conducted in five steps, consisting of different temperature and time sets: 130 °C/10min, 160 °C/15min, 170 °C/12min, 100 °C/7min, and 100 °C/3min. Mineral concentration determination was performed using the inductively coupled plasma optical emission spectrometer (ICP-OES) Optima 7000 DV (PerkinElmer, USA) with a radial configuration.

The Nutri-Score was calculated to determine the overall nutritional quality of the products. This calculation was performed using a table available on the French public health website (<https://www.santepubliquefrance.fr/media/files/02-determinants-de-sante/nutrition-et-activite-physique/nutri-score/tableur-calcul-nutri-score-en>)

### *Physical Characterization*

Rheology was performed to evaluate the yogurts' physical properties. A rotational rheometer (Bohlin Instruments, United Kingdom), coupled with a Peltier unit for temperature control, was used, and assays were conducted at 12 °C, in triplicate, using a cone-and-plate geometry probe ( $\alpha = 4$  degrees;  $d = 40$ mm). Samples were gently stirred for homogenization and then transferred to the measurement plate. Two distinct oscillatory assays were performed, namely, amplitude and frequency sweeps, in which the elastic (storage) and viscous (loss) moduli and the complex viscosity were assessed. Amplitude sweep was conducted at a constant frequency of 1 Hz, and strain varied from 0.001 to 0.1 Pa. Besides providing information regarding the different yogurt samples, this test allowed us to determine the linear viscoelastic region (LVER) of each sample type (LEY, NY, CY). The frequency was then varied (sweep) from 0.1 to 10 Hz, using a strain value that was required to be within the LVER of all the different yogurt types. Based on the results obtained in the amplitude sweeps, the strain value selected to be used in the frequency sweeps was 0.005.

### *Sensory Analysis*

The sensory analysis was performed in two phases. Firstly, a triangular test was performed by 14 trained panelists to identify if the addition of lupin flour (4%) could produce perceptible sensory alterations in the yogurt. For the triangular test, the two samples (LEY and NY) were presented to the panelists in sets of three, in identical containers coded with 3-digit random numbers. The samples were randomized, so each panelist had an equal chance of receiving any of the serving orders. The samples were presented in the order selected for each panelist who will be required to evaluate them from left to right. The results were analyzed using a one-tailed binomial test for significance, as a sample is known to be different. The number of tasters correctly identifying the different samples is calculated, and the total is tested for significance using a reference table (Watts *et al.*, 1989). After that, a quantitative descriptive sensory analysis was carried out by seven trained panelists. The panelists answered a specific questionnaire for set yogurt (CIDIL, 1995), adapted for the present study. The panel was instructed to score the intensity of attributes related to the samples' appearance, aroma, texture, and taste on a scale of 0 to 5, with 5 being the maximum score.

### *Statistical Analysis*

Statistical analyses were performed using the SPSS 17.0 software (SPSS, Chicago, IL, USA). The normality of the data was evaluated using Shapiro-Wilk's test. Data following normal distribution were analyzed using One-Way ANOVA coupled with Tukey's posthoc test when comparing the three samples (LEY, NY, and CY) or the t-student test when comparing two samples (LEY and CY). The data that did not comply with normality requirements were evaluated using the Kruskal-Wallis test associated with Dunn's posthoc test for three sample comparisons or the Wilcoxon-Mann-Whitney test in the case of two samples. The differences between means were considered significant for p-values below 0.05.

#### *3.2.3 Environmental impact assessment*

##### *Goal, scope, and boundary definition*

An LCA screening was undertaken for the LEY, NY, and CY for benchmarking purposes. A cradle-to-gate scope was considered, representing the production and distribution of all ingredients for the respective yogurts (Table 3.2), according to the ILCD handbook recommendation (European Commission, 2010). The manufacturing

process and end-of-life stages were assumed to be similar for all three products and were therefore excluded from the analysis. The open-source software OpenLCA v1.10.2 (GreenDelta, 2020) was used to calculate the environmental footprint of the three products, using product (ingredient) data from Agrifootprint 3.0 (Durlinger *et al.*, 2017) and Ecoinvent 3.7.1 (Wernet *et al.*, 2016) databases.

### *Functional units*

Considering that nutritional delivery is a primordial function of food, and therefore it needs to be considered to correctly assess the environmental impact (Miller *et al.* 2021), two functional units (FU) were used: 100 g of yogurt, and the Nutrient Density Unit (NDU), calculated as the formula below, developed by Van Dooren (2016). NDU is a useful formula because it requires few nutritional analyses and correlates with more extensive nutritional indices (Saget *et al.*, 2021).

$$NDU = \frac{\frac{EFA}{DV_{EFA}} + \frac{Protein}{DV_{Prot}} + \frac{Fibre}{DV_{fibre}}}{3 \times \frac{S_i}{2000 \text{ kcal}}}$$
(3.1)

Where:

*EFA* is the amount of essential fatty acids in 100g of product, expressed in grams.

*Protein* is the amount of protein in 100g of product, expressed in grams.

*Fiber* is the amount of fiber in 100g of product, expressed in grams.

*DV EFA* is the recommended daily value intake of essential fatty acids, expressed in grams = 12,4g†

*DV prot* is the recommended daily value intake of protein, expressed in grams = 50g\*

*DV fiber* is the recommended daily value intake of fiber, expressed in grams = 25g\*

*Si* is the amount of kilocalories in 100g of product, expressed in kilocalories.

\*Based on the US Food and Drug Administration's recommendations

†Based on the Institute of Medicine's DRI of EFA

### *Inventories*

The main differences between the LEY, NY, and CY were milk quantity and the presence of starches (Table 3.2). Ingredients data for the CY were collected from the

label, and quantities were estimated based on the nutritional composition shown. Global market product data were extracted from Ecoinvent v3.7.1, representing the average global market mix, therefore ensuring broad applicability. Some ingredients in Table 3.2 were not available in Ecoinvent v3.7.1, so proxies were used, notably: rice in place of rice semolina; chickpea in place of lupin flour; potato starch in place of pectin.

### *Impact Assessment*

The environmental footprints of the three yogurts were assessed across the sixteen environmental impact categories recommended in the Product Environmental Footprint (PEF) Category Rules Guidance (European Commission, 2018). To assist in the interpretation of environmental burdens across several categories, results were normalized by global person equivalents with the PEF recommended factors to generate comparable normalized scores across impact categories (European Commission, 2018).

## **3.3 Results and discussion**

### *3.3.1 Nutritional Characterization*

Table 3.3 shows the nutritional information and Nutri-Score of the three yogurts. Observing the general nutritional profile and the Nutri-Score, it is possible to notice that LEY presented advantages compared to the other two products. In the LEY, the addition of lupin flour enabled an increase in protein (40%), fiber (90%), and omega 3 (+353%) when compared to its natural counterpart (NY). According to European standards (Regulation EC N° 116/2010; Regulation EC N° 1924/2006), it is permitted to claim that LEY is “high in protein” (at least 20% of energy value provided by protein), “source of fibers” (at least 3g/100g) and “source of omega 3” (at least 0.3g of linolenic acid/100g). In fact, an increase in protein and dietary fiber is usually observed in food products enriched with pulses (Binou, *et al.*, 2020).

The high protein content (7g/100g) in the LEY yogurt allows offering the appropriate nutrient contribution for young children in smaller portions. Also, the fiber content can be an advantage since it can contribute to adequate fiber intake. Fiber is usually associated with children's health benefits, such as improving bowel function, preventing, and treating childhood obesity, maintaining normal blood glucose and blood pressure values, and reducing the risk of developing chronic diseases in the future (Korczak *et al.*, 2017). The presence of alpha-linolenic acid, an omega-3 fatty acid represents an additional gain. Several studies have linked the consumption of this

essential fatty acid with brain development and positive effects on cognitive development, especially in the first years of life (Shahidi and Ambigaipalan, 2018).

**Table 3.3** Nutritional information in 100g of lupin-enriched yogurt (LEY), natural yogurt (NY), and commercial yogurt (CY)

Energy/Nutrient	In 100 g of the product		
	LEY	NY	CY
Energy (kcal)	86	70	79
Total fat (g)	1.9	1.9	3.4
Saturated fat acids (g)	1.1	1.2	1.5
Unsaturated fat acids (g)	0.8	0.7	1.9
Omega 3 (mg)	53 <sup>†</sup>	15	< 10
Omega 6 (mg)	91	43	84.7
Carbohydrates (g)	9.3	7.7	8.9
Fiber (g)	1.9 <sup>*</sup>	1	1
Protein (g)	7 <sup>‡</sup>	5 <sup>‡</sup>	3
Moisture (g)	78.3	83.2	83
Ash (g)	1.6	1.2	0.6
Nutri-Score	A (-3)	A (-3)	B (0)

<sup>†</sup>Source of omega 3 [Regulation (EC) N° 116/2010]

<sup>\*</sup>Source of fibers [Regulation (EC) N° 1924/2006]

<sup>‡</sup>High in protein (per 100 kcal) [Regulation (EC) N° 1924/2006]

Table 3.4 shows the mineral composition of the three yogurts. Fe was not detected in any of the three samples, and Mg was just detected in the LEY. Zn, Mn, and Cu presented higher levels in the LEY, although the differences were not statistically

significant concerning NY. CY tended to show lower mineral levels compared with the other products, especially Zn, Mn, Ca, P, Na, and K ( $p < 0.05$ ).

**Table 3.4** Mineral composition of lupin-enriched yogurt (LEY), natural yogurt (NY), and commercial yogurt (CY)

Mineral	mg/L		
	LEY	NY	CY
Fe	<i>nd</i>	<i>nd</i>	<i>nd</i>
Zn	0.18±0.01 <sup>b</sup>	0.16±0.00 <sup>b</sup>	0.09±0.00 <sup>a</sup>
Mg	5.03±0.61 <sup>b</sup>	4.76±0.08	2.15±0.33 <sup>c</sup>
Mn	0.95±0.11	<i>nd</i>	<i>nd</i>
Cu	0.01±0.00	0.005±0.00	0.007±0.00
Ca	43.68±4.16 <sup>b</sup>	50.41±1.25 <sup>b</sup>	25.51±0.86 <sup>a</sup>
P	31.04±4.66	36.86±0.45 <sup>b</sup>	20.66±0.42 <sup>a</sup>
Na	13.83±1.67 <sup>b</sup>	17.48±0.54 <sup>b</sup>	9.11±0.36 <sup>a</sup>
K	67.48±7.75 <sup>b</sup>	71.87±2.52 <sup>b</sup>	37.43±1.36 <sup>a</sup>

*nd* - not detected

<sup>a</sup> significant difference ( $p < 0.05$ ) according to Tukey's posthoc test.

<sup>c</sup> significant difference ( $p < 0.05$ ) according to Dunn's posthoc test.

Although lupin is rich in minerals (Ruiz *et al.*, 2019), the mineral variation in LEY was not significant compared with NY, probably because lupin addition was not enough to promote an increase in mineral content. On the other hand, LEY presented a better mineral profile compared with CY. When discussing improved food mineral profile by adding pulses, bioavailability is always a question to be considered. In the case of lupin, an experimental study with rats found high bioavailability of P, Ca, and Mg in diets composed of lupin (*Lupinus albus*) flours (Porres *et al.*, 2006). Nevertheless, more bioavailability studies are necessary to make conclusions about the presented formulation.

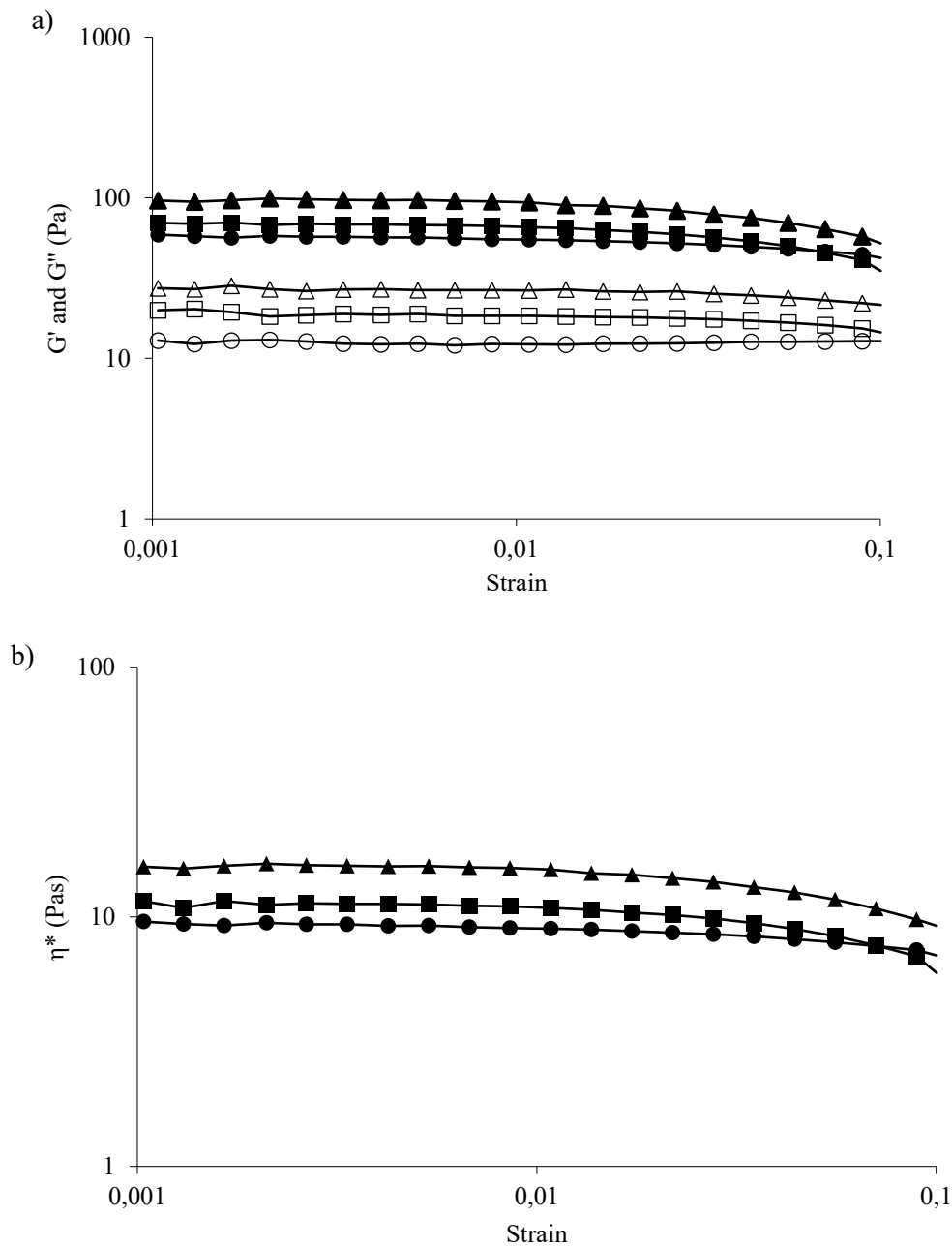
Despite nutritional benefits, using pulses in food formulations can present some limitations that need to be circumvented. One of the considerable challenges is the presence of anti-nutritional factors (Bresciani and Marti, 2019). One of the main disadvantages of adding lupin to products would be alkaloids, the allergic potential of this food (kouris-Blazos and Belski, 2016), and the presence of oligosaccharides that may cause flatulence such as  $\alpha$ -galactosides (Martínez-villaluenga *et al.*, 2008). Regarding alkaloids, their presence represents a concern for safe consumption since their ingestion can cause intoxication. The domestication process selected low alkaloid varieties for animal and human consumption (Ruiz *et al.*, 2019). In addition, strategies such as soaking, cooking or fermenting, and dilution by incorporating another food matrix reduce alkaloids to safe levels in the final product (Singh, 2017). Hence, the risks associated with these substances are reduced in LEY yogurt. Concerning the allergic potential, the consumption of LEY should not be recommended to those who are allergic to peanuts since they are more likely to develop a reaction to the lupin (kouris-Blazos and Belski, 2016). In relation to the oligosaccharides, the fermentation process can sharply reduce their content in the yogurt (Binou *et al.*, 2020; Martínez-villaluenga, *et al.*, 2008), and the remanecents compounds may be beneficial for the gut microbiota (Gullón *et al.*, 2015).

### 3.3.2 Physical Characterization

The storage modulus ( $G'$ ), the loss modulus ( $G''$ ), and the complex viscosity ( $\eta^*$ ) determined for all three yogurts (LEY, NY, and CY) are presented in Figure 3.2 (a and b, respectively). Rheological properties of experimental and control yogurt samples revealed important physical differences with respect to total solids content and nature. As shown in Figure 3.2a, the yogurt with skimmed milk powder (6%) and lupin flour (4%) added (LEY) showed the highest values of  $G'$  and  $G''$  compared with the other two control yogurts without lupin flour (NY, CY) and with a lower skimmed milk powder content (3%) (CY). Similar behavior was observed concerning  $\eta^*$  (Fig.3.2 b).

The elastic modulus ( $G'$ ) is related to the stiffness of the network and translates the solid-like characteristics of the sample, while the viscous modulus ( $G''$ ) is related to the viscous component of the yogurt and reflects liquid-like features (Steffe, 1996). The amplitude sweep allows the determination of the LVER, the region in which the viscoelastic properties are not dependent on the strain applied and, consequently, the elastic and viscous moduli are constant throughout. Results showed that the LVER was narrower in the yogurts with higher  $G'$  and  $G''$ , since the point when those parameters

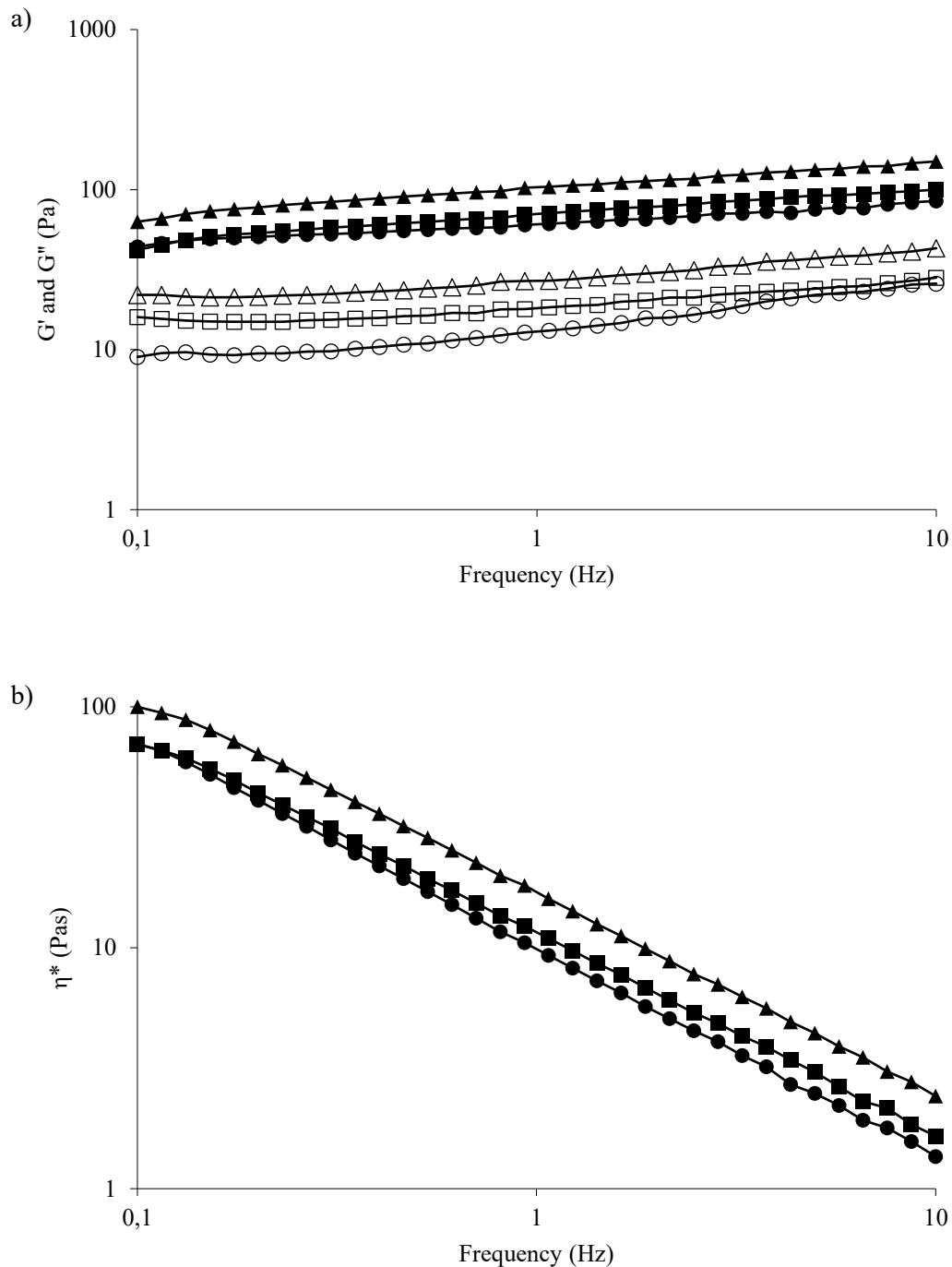
began to decrease occurred at a lower strain than in the remaining yogurts. The largest LVER was reported for CY since the corresponding  $G'$  began to decrease at a higher strain than the other yogurts ( $G''$  remained constant throughout the entire amplitude sweep). Complex viscosity also varied similarly, with a decrease starting earlier in LEY, followed by NY, and finally CY, which was the last to begin to decrease. In order to perform frequency sweep tests, a strain value within the LVER must be selected and, as such, 0.005 was the strain chosen.



**Figure 3.2** (a) Elastic and viscous moduli (filled symbols -  $G'$ ; open symbols -  $G''$ ) and (b) complex viscosity ( $\eta^*$ ) of the lupin-enriched LEY ( $\Delta$ ), natural NY ( $\square$ ), and commercial CY ( $\circ$ ) yogurts.

The dependence of  $G'$  and  $G''$  and  $\eta^*$  on frequency is presented in Figure 3.3a and Figure 3.3b, respectively. When varying the frequency, for all three yogurts, both moduli increased with increasing frequency, and  $G'$  was always higher than  $G''$  (Fig. 3.3a), which reflects a solid-like behavior of the samples. As such, the yogurts can be characterized as being viscoelastic solids. Such trend, regardless of lupin/skimmed milk supplementation, denotes a weak viscoelastic behavior characteristic of yogurt, in general. Furthermore, both  $G'$  and  $G''$  increased with increasing frequency. Results showed significant differences ( $p < 0.05$ ) between the yogurts prepared with the distinct formulations. Regarding all parameters, significantly ( $p < 0.05$ ) higher values were obtained for LEY samples than for NY and CY, which means that LEY presents/detains a more prominent solid-like behavior. Concerning NY and CY, only  $G''$  presented significant differences ( $p < 0.05$ ) between the two types of yogurts, while both  $G'$  and  $\eta^*$  did not ( $p > 0.05$ ). This indicates similarity between the yogurts in terms of physical properties.

Concerning complex viscosity (Fig. 3.3b), values decreased with increasing frequency, which was expected for such matrices, as at higher frequencies, the matrices become more fluid.



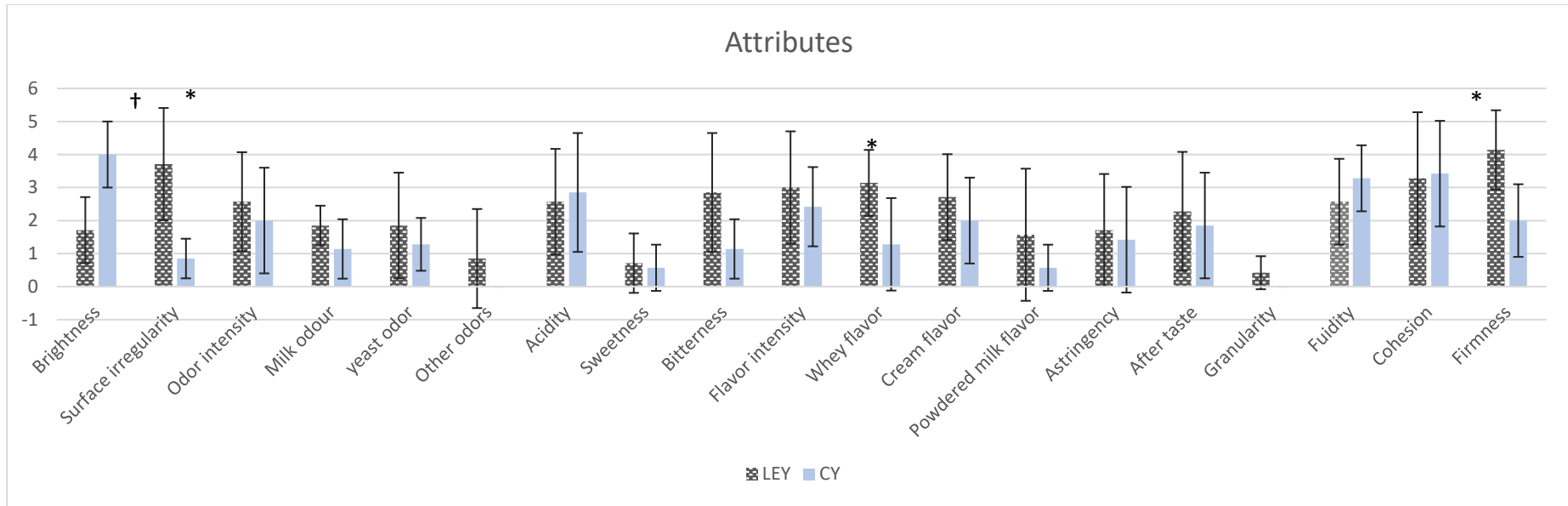
**Figure 3.3** (a) Elastic and viscous moduli (filled symbols -  $G'$ ; open symbols -  $G''$ ) and (b) complex viscosity as a function of frequency of the lupin-enriched LEY ( $\Delta$ ), natural NY ( $\square$ ), and commercial CY ( $\circ$ ) yogurts.

The results evidenced that LEY was the stiffest yogurt with the highest viscosity, whereas NY presented intermediary results, and CY presented the most increased fluidity and lowest viscosity. Yogurt structure is strongly influenced by the total solids content, especially in what concerns protein content. An increase in protein content results in linear increases in the elastic modulus and apparent viscosity (Divyang *et al.*, 2016). The

supplementation of yogurt with skimmed milk powder and with lupin flour led to a higher total solids content which increased the capacity to establish crosslinking bridges between milk casein micelles and other added proteins. In fact, some components present in lupin and other pulses, such as proteins with a more significant number of hydroxyl groups and fiber, have a higher capacity for water binding, which is related to viscosity (Bresciani and Marti, 2019; Villarino *et al.*, 2016). These components also give greater stability to foods, improving product shelf-life (Foschia *et al.*, 2017). In a recent study, Saleh *et al.* (2020) found that chickpea starch is a good stabilizer used in low-fat yogurts, maintaining the texture after 15 days of storage. Similarly, Raza *et al.* (2022) showed that the addition of 3-5% roasted chickpea flour to yogurt milk improved final product physicochemical, rheological, and sensory characteristics while providing essential nutrients of value for several target groups. In the case of the developed LEY yogurt, additional studies are required to evaluate formula stability during storage.

### 3.3.3 Sensory Analysis

The triangular test revealed no significant sensory differences between the NY and LEY. Thus, the descriptive sensory test was performed with LEY and CY. Figure 3.4 displays the intensity of sensory attributes in both yogurts. Regarding appearance, CY was brighter ( $p < 0.05$ ) and had a more regular surface ( $p < 0.05$ ) than LEY. Yogurts showed similar scores for most attributes in terms of smell and taste, except for whey flavor which tended to be higher in LEY ( $p < 0.05$ ). Although without statistical significance, LEY also presented more bitterness, granularity, and after-taste, which are unexpected attributes in the case of yogurts. Concerning texture parameters, LEY tended to be less fluid and firmer, showing a more significant difference in the latter attribute ( $p < 0.05$ ). These results agree with the rheological parameters.

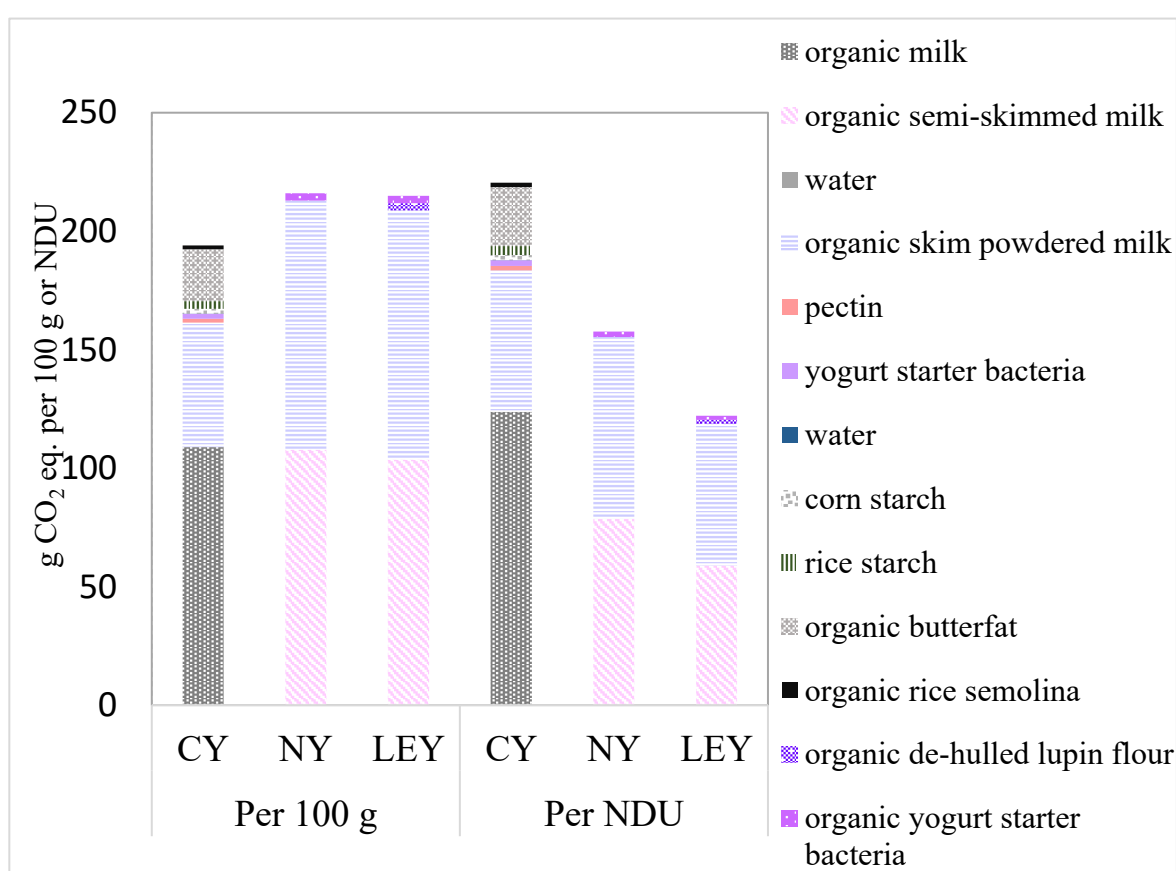


**Figure 3.4** Quantitative descriptive analysis of sensory attributes of the lupin-enriched (LEY) and commercial (CY) yogurts. The values presented correspond to the average score of each characteristic.  
 \*Significant difference ( $p < 0.05$ ) according to Wilcoxon-Mann-Whitney test.  
 †Significant difference ( $p < 0.05$ ) according to t-student test.

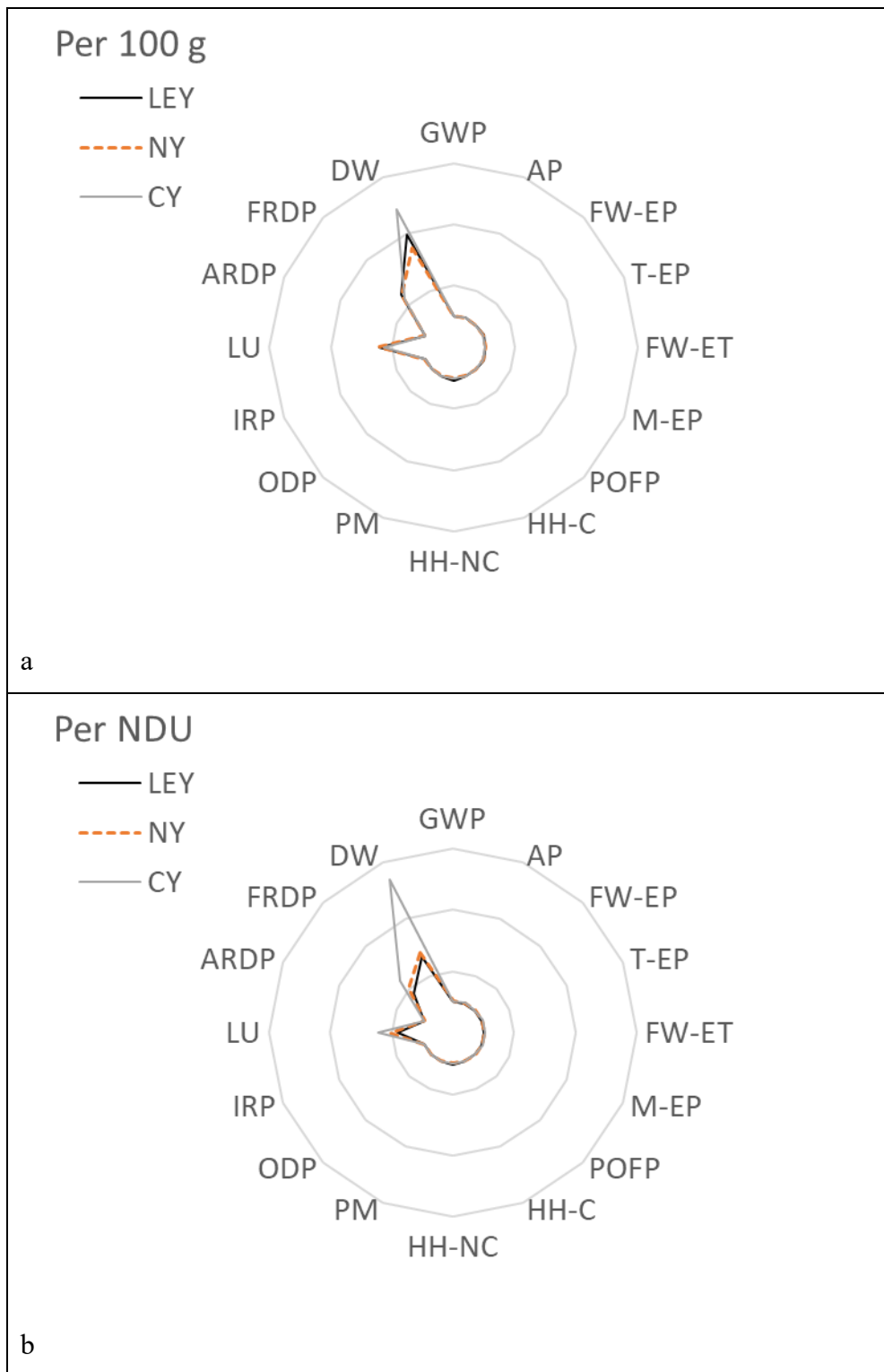
A significant limitation to adding lupin flour to food products is its sensory acceptability (Bresciani and Marti, 2019). Additionally, a persistent after-taste is a recurrent problem in products with lupin, which is the primary sensory obstacle to using it in different formulations (Villarino *et al.*, 2016). The residual alkaloids and the formation of Maillard products during the heating of amino acids and sugars can be related to this finding. A detailed problem analysis, followed by adaptations in the processing, may outline sensory questions (Singh, 2017). The use of more refined ground flour, a rigorous time and temperature control during processing, and a deep chemical evaluation to observe undesirable compounds in order to reduce, or even remove them, are measures that can be adopted to minimize undesirable characteristics. Furthermore, some consumers are more likely to choose a product perceived as more health, even if it presents some sensory defect (Maruyama *et al.*, 2021).

### 3.3.4 Environmental impact

The global warming potential (GWP), or “carbon footprint”, of a serving (100g) of LEY is similar (1% smaller) than for the NY, but 11% larger than for the CY (Fig. 3.5), largely because of a greater input of powdered milk (5.4 vs 2.7 g per 100 g). However, owing to higher nutrient density, the LEY carbon footprint is approximately half that of the CY per NDU, and 23% smaller than for the NY on an NDU basis (Fig. 3.5). Despite a wide range of ingredients, especially for the CY, footprints are dominated by milk-derived products: organic milk, organic semi-skimmed milk, skim milk powder, and organic butterfat.



**Figure 3.5** Contribution of ingredients to global warming potential expressed as g CO<sub>2</sub> eq. per 100 g or per NDU, for the commercial (CY), natural (NY), and lupin-enriched (LEY) yogurts



**Figure 3.6** Normalized environmental scores across 16 impact categories for the lupin-enriched (LEY), natural (NY), and commercial (CY) yogurts, expressed per 100 g of yogurt (a), and per NDU (b). Impacts are shown, clockwise from the top: global warming potential (GWP), acidification potential (AP), freshwater eutrophication potential (FW-EP), terrestrial eutrophication potential (T-EP), freshwater ecotoxicity (FW-ET), marine eutrophication potential (M-EP), photochemical ozone formation potential (POFP), human health cancer & non-cancer effects (HH-C & HH-NC), particular matter (PM), ozone depletion potential (ODP), ionizing radiation potential (IRP), land use (LU), abiotic resource depletion potential (ARDP), fossil resource depletion potential (FRDP), and dissipated water (DW).

The Environmental Footprint impact assessment methodology applied generates results across 16 impact categories. Figure 3.6 shows the normalized scores (divided by per capita burdens) across all categories. Per 100 g serving, LEY and NY normalized scores are similar across almost all impact categories, whilst the CY has slightly lower normalized scores than the LEY for all impact categories except for dissipated water (DW), where rice starch and rice-derived semolina contribute towards 40% of the DW footprint for CY. Normalized scores for DW burdens are at least an order of magnitude higher than for other impacts, suggesting that the ingredients considered here make a large contribution to water consumption compared with other food and activities – via bovine production and rice cultivation. When expressed per NDU, the LEY has lower normalized scores than both the NY and the CY yogurts across all categories, apart from the human-health non-cancer category where NY has the lowest score (Fig. 3.6).

The LCA screening undertaken here indicates that LEY has a similar environmental footprint to NY, but a slightly higher environmental footprint compared with CY, per 100 g serving, because of the higher skimmed milk powder content. Evaluating the environmental impact of yogurt production González-García *et al.* (2013) also found a remarkable contribution of milk powder in the main impact categories. On the other hand, the higher protein, fiber, and fatty acid contents of LEY result in a smaller environmental impact per unit of nutritional density compared with the other yogurts, especially compared with the CY – corroborating other recent assessments of legume-derived products (Saget *et al.*, 2020; 2021). Proxy data were required for some ingredients, introducing some uncertainty. As an alternative, it could be possible to reduce to some extent the percentage of skimmed milk powder in LEY, reducing the environmental impact without significantly compromising nutritional, physicochemical, and sensory aspects. In any case, the greatest contribution to improving understanding of the environmental sustainability of LEY would be to consider the crop rotation changes that could be associated with new large-scale demand for lupin driven by LEY commercialization. Past work has indicated that legume grains can play an important role in driving the overall environmental sustainability of entire crop rotations by, *inter alia*, delivering biologically fixed nitrogen to following crops and acting as a break crop (Costa *et al.*, 2021). In further evaluation, a consequential LCA of commercial LEY deployment could consider which kinds of crop rotations would be modified by this increased demand for lupin, alongside the consequences of reduced demand for, *inter alia*, rice-derived products.

### **3.4 Conclusion**

The addition of lupin flour to yogurt conferred nutritional advantages to the product. Also, lupin flour adds more rigidity and viscosity to the product. There were no significant sensory differences between LEY and NY, and most of the sensory attributes of LEY were similar to those of CY. However, undesirable sensory characteristics such as bitterness, granularity, and after-taste were observed. These may be overcome with processing adaptations such as using more refined ground flour and rigorous time and temperature control during processing. Further studies are required to evaluate mineral bioavailability, the presence of antinutritional factors, and formula stability during storage. Regarding environmental impact, LEY has similar normalized scores to NY and slightly worse normalized scores compared with CY for most impact categories per 100 g serving. However, environmental scores for LEY are better than for the other yogurts when expressed per unit of nutrition. Using lupin flour to enrich yogurts for children can be an alternative to producing more nutritious and functional products. Infant commercial formulations would benefit using lupin to replace some texture ingredients.

#### **Chapter 4. Metabolic evaluation of lupin-enriched yogurt by nuclear magnetic resonance metabolomics**

The yogurt developed in Chapter 3 was evaluated using nuclear magnetic resonance (NMR) spectroscopy with the aim of characterizing the metabolic adaptations of lupin-enriched yogurt during fermentation. This chapter is an original article published in the Journal of Agricultural and Food Chemistry:

Rodrigues JA; **Ferro E**; Araújo R; Henriques AV; Gomes AM; Vasconcelos MW; Gil AM. 2024. Metabolic Evaluation of Lupin-Enriched Yogurt by Nuclear Magnetic Resonance Metabolomics. *Journal of Agricultural and Food Chemistry* 72(1), 894-903

## ABSTRACT

Untargeted nuclear magnetic resonance (NMR) metabolomics was used to evaluate compositional changes during yogurt fermentation upon lupin enrichment compared to traditional conditions. Lupin significantly changed the sample metabolic profile and its time course dynamics, seemingly delaying microbial action. The levels of organic and amino acids were significantly altered, along with those of some sugars, nucleotides, and choline compounds. Lupin seemed to favor acetate and formate synthesis, compared to that of citrate and fumarate; a higher formate levels may suggest increased levels of *Streptococcus thermophilus* action, compared to *Lactobacillus bulgaricus*. Lupin-yogurt was poorer in hippurate, lactose (and hence lactate), galactose, glucose-1-phosphate, and galactose-1-phosphate, containing higher orotate levels (possibly related to increased uridine derivatives), among other differences. Trigonelline was confirmed as a lupin marker, possibly together with glutamate and histidine. Other metabolite trajectories remained unchanged upon lupin addition, unveiling unaffected underlying processes. These results demonstrate the usefulness of untargeted NMR metabolomics to understand/develop new foodstuffs and their production processes, highlighting the identity of a variety of bioactive metabolites with importance for human health.

**Keywords:** Fermentation, yogurt, lupin, legumes, metabolomics, nuclear magnetic resonance spectroscopy.

## 4.1 Introduction

Yogurt is one of the most popular fermented dairy products, the consumption of which is increasing worldwide due to its unique flavor, high nutritional value, and associated health benefits (e.g., digestion enhancement, immunostimulatory effects, and anticarcinogenic and antidiabetic properties) (Chandan *et al.*, 2017). Yogurt is obtained from fermenting milk, usually using the two symbiotic species of lactic acid bacteria, *Streptococcus thermophilus* (ST) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB) as starter cultures (Corrieu and Béal, 2016). In recent years, consumers have become increasingly aware of the health-related functions of foods, and this has led to a higher demand for products with improved nutritional value and health benefits. Hence, several approaches have been followed to improve yogurt, such as the inclusion/improvement of probiotics (Peng *et al.*, 2022) and the incorporation/addition of fruit and/or other plant-based ingredients (Ahmad *et al.*, 2022). Regarding the latter, legumes appear as interesting potential ingredients due to their benefits related to reduced risk of cardiovascular diseases (e.g., obesity and type-2 diabetes), improvement of gut microbial diversity, modulation of oxidative stress and inflammatory status, and reduced risk of cancer (Geraldo *et al.*, 2022). Lupin (*Lupinus albus*) is a pulse crop that grows in the Mediterranean region, which contains relatively high contents of protein and other nutrients such as fiber and polyunsaturated fatty acids (often missing in conventional formulations), while exhibiting beneficial antioxidant and prebiotic properties. The main antinutritional factors found in sweet lupin comprise raffinose family oligosaccharides, phytic acid, and polyphenols, in tandem with relatively low amounts of lectins and trypsin inhibitors, compared to other legumes such as cowpea, kidney bean, and soybean (Kefale *et al.*, 2022). Lupin addition to yogurt has been evaluated for nutritional, rheological, and sensorial properties (Vieira *et al.*, 2022), having revealed beneficial effects, despite slightly undesirable sensorial characteristics (bitterness, granularity, and after-taste). Lupin-enriched yogurt was revealed to contain increased levels of protein (+40%), fiber (+90%), and omega-3 fatty acids (+353%) in relation to its natural yogurt counterpart, thus enabling differential nutritional claims (such as “high in protein”, “source of fiber”, and “source of omega-3”) and supporting previously mentioned health benefits. Furthermore, the addition of lupin enhanced yogurt texture attributes, generating a firmer, more consistent, and adhesive matrix, compared to natural yogurt.

However, only a few studies have measured detailed metabolic changes upon legume addition to foods in general. To this end, food untargeted metabolomics appears

as an excellent approach for product characterization and classification based on metabolic profile (Cubero-Leon *et al.*, 2014). Originally applied in health-related areas, metabolomics combines high-throughput analytical information through nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry methods with multivariate statistical analysis. NMR provides simultaneous information on several different compound families, requiring minimal sample preparation and offering rapidity and high reproducibility. The method has been applied to many foods, e.g., beer, coffee, fruit juices, olive oil, tea, vinegar, wine, and dairy products (Cubero-Leon *et al.*, 2014; Wu *et al.*, 2022). In yogurt, metabolomics has been used to assess the impact of different probiotic strains, nutritionally rich constituents and different experimental/technological conditions on yogurt metabolic composition, nutritional quality, and physicochemical and organoleptic characteristics (Peng *et al.*, 2022; Lopes *et al.*, 2020; Li *et al.*, 2023). The most studied topic has been the use of different strains with probiotic properties, the majority belonging to the *Lactobacillus*, *Bifidobacterium*, and *Levilactobacillus* genera (Peng *et al.*, 2022; Settachaimongkon *et al.*, 2016; Settachaimongkon *et al.*, 2014). Also, different fermentation temperatures have been tested, either alone (Jia *et al.*, 2021) or combined with probiotic strains (Wang *et al.*, 2021), anaerobic conditions (Ding *et al.*, 2022), or high-pressure technology (Lopes *et al.*, 2020). In addition, since  $\gamma$ -aminobutyric acid (GABA) is suggested to reduce stress, inhibit cancer cell proliferation, decrease blood pressure, and prevent diabetes, GABA-enriched yogurt has recently attracted considerable interest (Fan *et al.*, 2023). In the case of plant- and/or fruits-based yogurts, metabolomics has studied the effects of added highland barley (Li *et al.*, 2023), hemp seeds (Xu *et al.*, 2023), and red-purple pitaya fruit colorant (Lima *et al.*, 2020). Regarding legumes, and lupin in particular, the presence of antinutrient compounds (Geraldo *et al.*, 2022) makes it even more important to fully analyze the effect of incorporating such an ingredient in popular foodstuffs.

In this work, the impact of the addition of lupin during set-yogurt fermentation on the metabolic profile of yogurts was assessed by NMR metabolomics, building up on a previous report of beneficial nutritional, sensorial, and environmental impact characterization of the same product (Vieira *et al.*, 2022). The metabolic profiles of lupin-enriched samples were evaluated as a function of fermentation time (0, 2, 4, and 6 h and postrefrigeration, Pr), and compared with those of yogurts produced under control conditions, providing insights into the role of lupin on fermentation biochemistry and final product composition.

## 4.2 Experimental section

### 4.2.1 Yogurt preparation

The yogurts were prepared as described previously (figure 3.1). Briefly, for all yogurts, 6% of skimmed milk powder (Molico powdered milk, 6.8 g/100 mL) was mixed with semiskimmed milk (Milhafre, Azores, Portugal). The lupin-enriched formulation included the addition of 4% dehulled organic fine-ground lupin flour (4.16 g/100 mL) (Provida, Portugal). The mixture was homogenized and heated to  $90 \pm 2$  °C, in a Thermomix (Vorwerk, Germany), for 3.5 min. After cooling to  $40 \pm 2$  °C, the preparation was inoculated with an organic powder preparation for yogurt (Natali, France) containing *Streptococcus thermophilus* (ST) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB) and homogenized (2 min). The fermentation step occurred for approximately 6 h (until pH of  $4.6 \pm 0.1$  was reached), at  $42 \pm 2$  °C, under complete rest. After fermentation, the yogurts were cooled down and maintained at 4 °C for 24 h (refrigeration step, figure 3.1), after which the samples were stored at  $-80$  °C, until analysis. Overall, a total of 30 samples were obtained (15 controls and 15 lupin-enriched samples) distributed by five different time intervals during fermentation (0, 2, 4, and 6 h and Pr, with  $n=3$  per time point). Each sample (30–50 g) was immediately stored at  $-80$  °C.

### 4.2.2 NMR Spectroscopy

For NMR analysis, yogurt samples were prepared as described in a previous report (Lemos *et al.*, 2022). Briefly, after thawing, 10 g of each yogurt sample was centrifuged (6500g, 10 min, room temperature). After centrifugation, 4 mL of the supernatant was collected, again centrifuged (6500g, 10 min, room temperature) and left to precipitate overnight at 4 °C. Three mL of the supernatant were filtered (acetate membrane,  $\phi$  0.22  $\mu$ m) yielding ca. 1.5 mL of sample. The filtered samples were dried under a vacuum and stored at  $-80$  °C until NMR analysis.

For analysis, the extracts were reconstituted using 700  $\mu$ L of deuterated 0.1 M phosphate buffer at pH 7.4, containing 0.25% sodium 3-trimethylsilyl-2,2,3,3-d<sub>4</sub>-propionate (TSP) for chemical shift referencing. The mixture was then vortexed, centrifuged (8000g, 10 min, room temperature), and a total volume of 550  $\mu$ L was transferred into 5 mm NMR tubes. All NMR spectra were recorded on a Bruker AVANCE III 500 spectrometer (Bruker, Rheinstetten, Germany) operating at 500.13 MHz frequency for <sup>1</sup>H observation, equipped with a 5 mm TXI, at 298 K. For each sample, a

standard 1D  $^1\text{H}$  NMR spectrum was recorded using the *noesypr1d* pulse program (Bruker library) with 2.3 s of acquisition time, 4 s relaxation delay, 512 scans, 7002.8 Hz spectral width, and 32 k data points. Each free-induction decay was zero-filled to 64 k points and multiplied by a 0.3 Hz exponential line-broadening function prior to Fourier transform. Spectra were manually phased, baseline corrected, and chemical shift referenced to the TSP signal ( $\delta$  0.0). 2D NMR spectra ( $^1\text{H}/^1\text{H}$  total correlation, TOCSY,  $^1\text{H}/^{13}\text{C}$  heteronuclear single quantum correlation, HSQC, and *J*-resolved) were recorded to aid spectral assignment. Peak assignment was also based in existing literature (Settachaimongkon *et al.*, 2016; Settachaimongkon *et al.*, 2014; Lemos *et al.*, 2022; Trimigno *et al.*, 2020) and spectral databases, namely, Bruker BIOREFCODE, the FooDB project ([www.foodb.ca](http://www.foodb.ca), accessed in 10-05-2023), the human metabolome (HMDB) (Wishart *et al.*, 2018), and the Chenomx Profiler SOFTWARE (Chenomx NMR Suite 7.5, Chenomx Inc., Edmonton, Canada).

#### 4.2.3 Statistical analysis

For multivariate analysis (MVA), the full resolution (1D)  $^1\text{H}$  NMR spectra were converted into data matrices (AMIX-viewer 3.9.14, Bruker Biospin, Rheinstetten, Germany), excluding the suppressed water region ( $\delta$  4.77–4.83). Spectra were aligned using recursive segment-wise peak alignment (Matlab 8.3.0, The MathWorks Inc., Natick, Massachusetts, USA) (Veselkov *et al.*, 2009). Since the overall spectral profile changed significantly during fermentation due to changes in intense and broad lipid signals and thus affecting total spectral area (Figure S2), spectra were normalized using the probabilistic quotient normalization (PQN) (Dieterle *et al.*, 2006), taking the average of all spectra for 0 h of fermentation as reference (PQN normalization was carried out using Matlab 8.3.0, the MathWorks Inc., Natick, Massachusetts, USA). Principal component analysis (PCA) and partial-least-squares discriminant analysis (PLS-DA) were applied, upon unit variance (UV) scaling (SIMCA P11.5, Umetrics, Umea, Sweden) and models were considered robust for predictive power ( $Q^2$ ) values approaching or above 0.5. The MVA results were visualized through factorial coordinates (score plots) and factorial contributions (loading plots). The loading plots were back transformed by multiplying each variable by its standard deviation and colored according to each variable importance to the projection (VIP) value (Matlab 8.3.0, The MathWorks Inc., Natick, Massachusetts, USA). For each identified metabolite or relevant resonance, peak areas were integrated in the raw spectra (AMIX-viewer 3.9.14, Bruker Biospin, Rheinstetten,

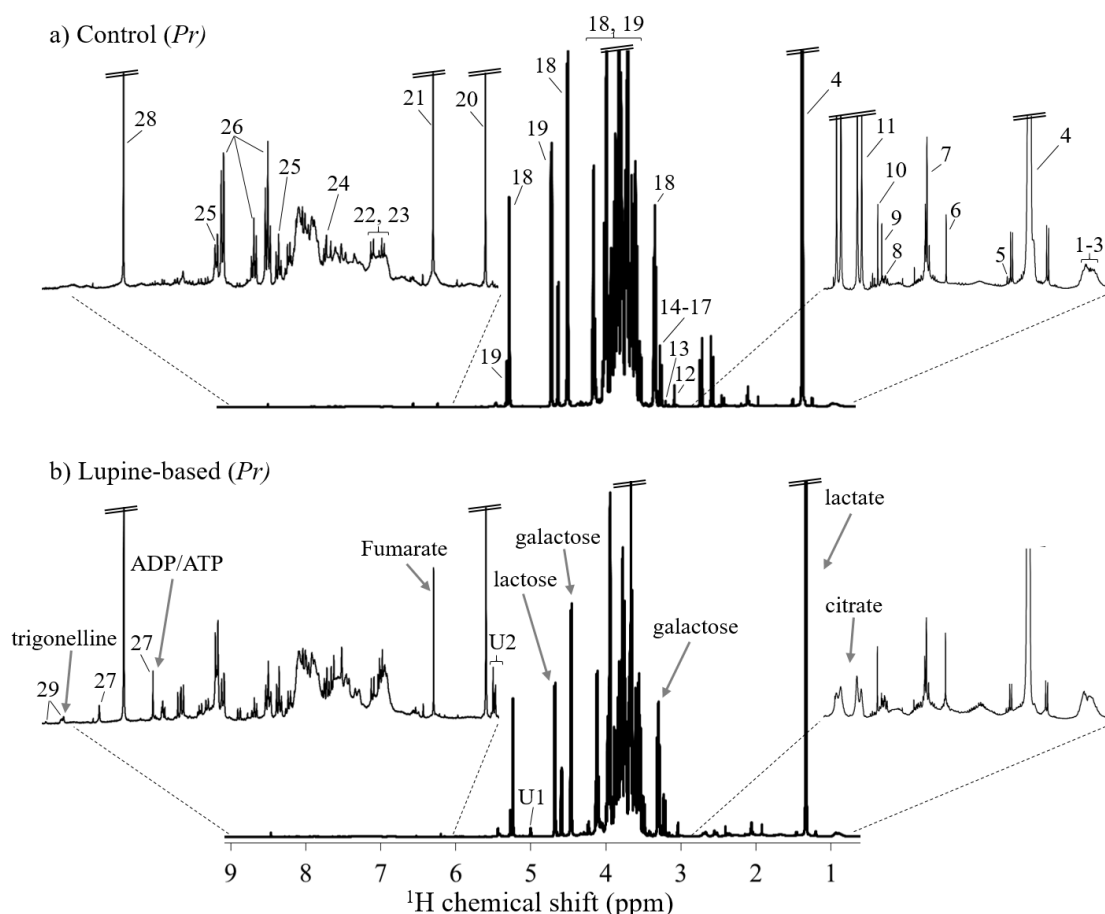
Germany) and normalized by PQN. For each pairwise comparison, effect size (ES) values (Berben et al., 2012) (considering  $|ES|$  values  $>2.0$  of relevant magnitude) and statistical significance ( $p$ -values) were assessed by the Wilcoxon test (R software, version 4.0.0, combined with R-studio version 1.3.1093, R Foundation for Statistical Computing, Vienna, Austria). The Benjamini and Hochberg false discovery rate (FDR) (Benjamini and Hochberg, 1995) correction was applied for multiple testing.

## 4.3 Results

### 4.3.1 $^1H$ NMR spectra of yogurts

The average  $^1H$  NMR spectrum of control yogurts (PrC, Figure 4.1a) reflects the high complexity of the samples in terms of metabolite composition. Peak assignment unveiled a total of 44 metabolites (Table S1), comprising: 12 organic acids; 13 amino acids and derivatives; 7 sugars and derivatives, including uridine derivative UDP-galactose to add to other 4 nucleotides and derivatives (adenosine monophosphate and/or triphosphate (ADP/ATP), uridine and uridine monophosphate (UMP)); choline, glycerophosphocholine (GPC), and phosphocholine (PC); and other metabolites (namely, acetoin, acetylcarnitine, dimethylsulfone and tyrosol). This set of identified compounds is broadly consistent with previous NMR studies of yogurts (Settachaimongkon *et al.*, 2016; Settachaimongkon *et al.*, 2014) and milk products in general (Lemos *et al.*, 2022).

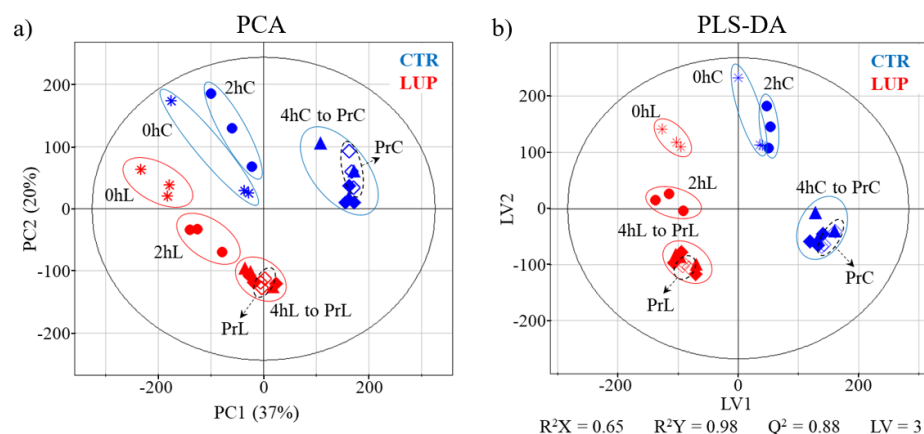
The average  $^1H$  NMR spectrum registered for control conditions (Figure 4.1a) is dominated by resonances arising from sugar metabolites, particularly lactose (peaks 18) and galactose (peaks 19), and lactate (peak 4). Visual comparison with the spectrum of lupin-enriched yogurt reveals the presence of the same set of compounds detected (with the exception of trigonelline, as discussed below) but in distinct relative levels (Figure 4.1b). Inspection unveils apparent lower amounts of lactose (peaks 18), galactose (peaks 19); citrate, fumarate, hippurate and lactate, although the latter peak is cut-off in Figure 4.1 (peaks 11, 21 and 26 and 4, respectively); and higher amounts of ADP/ATP (peaks 27) (Figure 4.1b). Notably, trigonelline (peak 29, Figure 4.1b), an alkaloid plant hormone found in several seeds and seedlings of legumes, including lupin (Martínez-Villaluenga *et al.*, 2006), was only detected in lupin-enriched yogurts. Additionally, some still unassigned resonances between *ca.*  $\delta$  5.0-6.2 (Figure 4.1b) were clearly increased in lupin-enriched yogurts. However, all the above visual changes between the two final products require statistical evaluation, as discussed below.



**Figure 4.1.** 500 MHz  $^1\text{H}$  NMR average spectra of yogurt corresponding to post-refrigeration after 6 hours fermentation, for: (a) control samples (traditional fermentation) and (b) lupine-enriched samples. Insets show the expanded aliphatic ( $\delta$  0.5-3.0) and aromatic regions ( $\delta$  6.0-10). Peak assignments: 1-3, aliphatic amino acids (1: isoleucine; 2: leucine; 3: valine); 4, lactate; 5, alanine; 6, acetate; 7, *N*-acetylglucosamine; 8, glutamate; 9, pyruvate; 10, succinate; 11, citrate; 12, creatine; 13, dimethyl sulfone; 14, acetylcarnitine; 15, choline; 16, phosphocholine, PC; 17, glycerophosphocholine, GPC; 18, lactose; 19, galactose; 20, orotate, 21, fumarate; 22, tyrosol; 23, tyrosine; 24, phenylalanine; 25, benzoate; 26, hippurate; 27, adenosine diphosphate and/or adenosine triphosphate, ADP/ATP; 28, formate; and 29, trigonelline. The full list of identified metabolites is shown in Table S1. Visually identified spectral variations between the different yogurts are highlighted by arrows and full metabolite names in (b).

#### 4.3.2 Multivariate analysis of control and lupin-enriched yogurts

PCA of the  $^1\text{H}$  NMR spectra of all control samples (Figure 4.2, left, blue symbols, positive PC2) revealed a gradual profile change up to 4 h, with subsequent overlap of 6 h and PrC samples. Similar results were observed for lupin-enriched samples (Figure 4.2, left, red symbols, negative PC1), although their clear separation from control samples, in both PCA and PLS-DA scores plots (Figure 4.2, left and right, respectively) indicates that significantly distinct profiles characterize each group of samples.



**Figure 4.2.** Multivariate statistical analysis of the  $^1\text{H}$  NMR spectra of yogurts produced under control conditions (blue, C, CTR) and with lupin enrichment (red, L, LUP), throughout the fermentation process. Symbols corresponding to fermentation time: 0 h (\*); 2 h (●); 4 h (▲); 6 h (◆); and post-refrigeration, Pr (◇). Scores scatter plots for (a) PCA and (b) PLS-DA.  $R^2X$ : fraction of the variation of X-variables explained by the model;  $R^2Y$ : fraction of the variation of Y-variables explained by the model; and  $Q^2$ , predictive power. Abbreviations: LV, latent variables; PC, principal components.

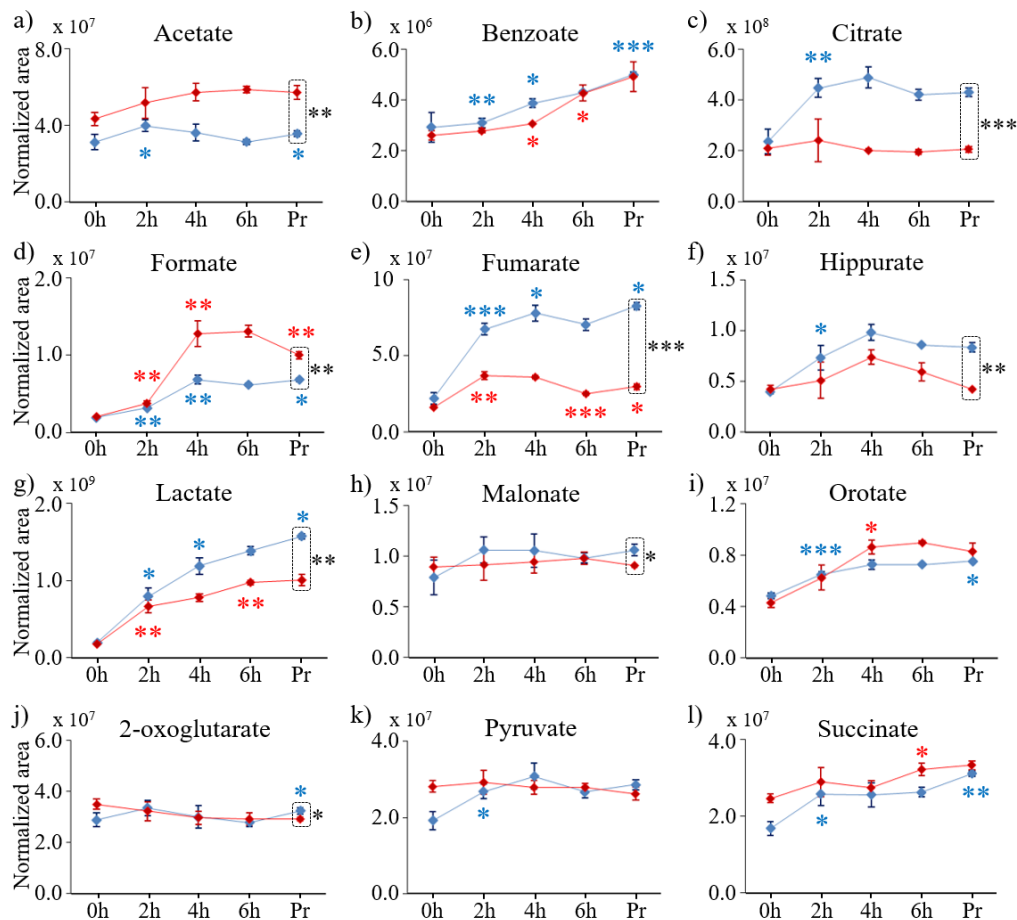
In both groups, the major differences observed between 0, 2 and 4 h samples in the full models (Figure 4.2) were clearly confirmed by PCA and PLS-DA of each group separately (Figure S3a and b), which also illustrates higher sample heterogeneity at 0 and 2 h. In the loadings plot (Figure S3c) corresponding to the PLS-DA model of lupin-enriched samples (Figure S3b, right), a set of positive broad resonances identifies lipids (Rodrigues *et al.*, 2023), as more abundant in 0 and 2 h samples (in positive PC1/LV1), compared to the remaining samples. Indeed, a gradual decrease in lipid resonances is clearly seen in the spectra of the 0 to 2 to 4 h samples, not only of lupin-enriched samples but also of control samples (Figure S3). In addition, in the same loadings plot (Figure S3c), the negative loadings profile indicates higher abundance of many metabolites (numbered as in shown in Figure 4.1) at 4 h, 6 h and PrC, including lactate (peaks 4) and sugars, among others. Furthermore, some compositional changes seem to be induced by refrigeration, particularly in control samples, for which PrC samples show some separation from 6 h samples, in PCA (Figure S3a).

#### 4.3.3 Metabolic changes during fermentation of control yogurt

Fermentation under control conditions is accompanied by changes in a total of 33 compounds, including many organic acids and amino acids, some sugars, nucleotide derivatives, choline compounds and other metabolites (Table S2). Changes are expressed in effect size, ES (Table S2) and represented in normalized area trajectories, as a function

of time in Figures 3-5. The changes noted between 0 and 2 h are the most significant, both in magnitude and significance (note that most variations remain significant upon FDR correction, \* in Table S2). Less number/magnitude of changes occur from 2 to 6 h (with the 4-6 h period appearing almost eventless), followed by a large set of changes upon refrigeration.

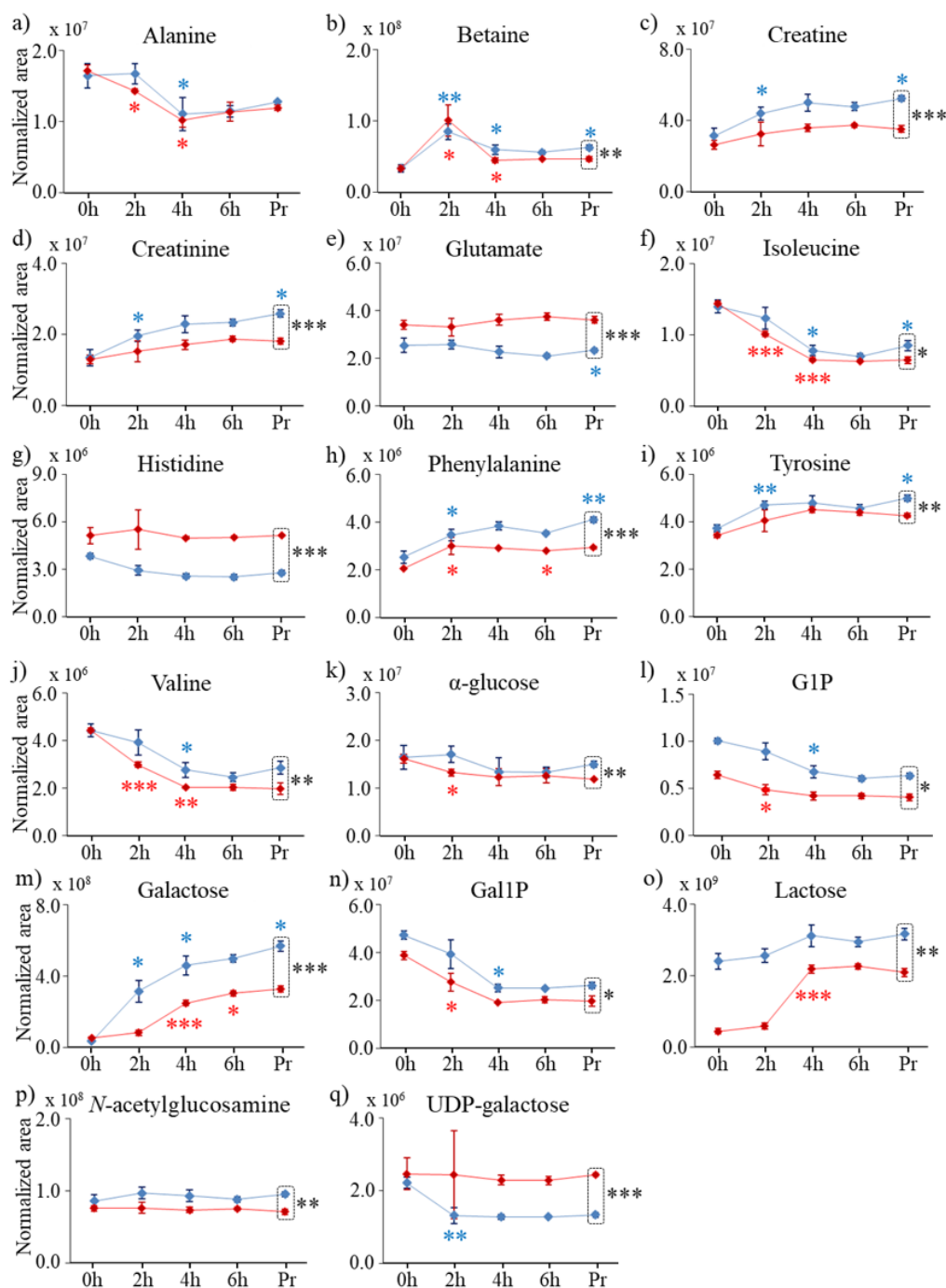
Regarding organic acids, changes include major initial increases in lactate (effect size, ES = 34), followed by hippurate (ES = 11) and formate (ES = 10) (Table S2). Indeed, a general increase is observed in several organic acids, although with distinct dynamics (Figure 4.3, blue trace; and Table S2): i) steady increases in benzoate and lactate, ii) citrate, hippurate, orotate, pyruvate and succinate reaching a plateau at 2 h, and iii) formate and fumarate reaching maximum values at 4 h. Acetate, malonate and 2-oxoglutarate show little variation throughout the process. Refrigeration leads to increases in most of the acids detected, with ES ranging between 2.3 (formate) and 7.1 (benzoate), except for citrate, hippurate and pyruvate, which do not vary (Table S2).



**Figure 4.3.** Organic acids trajectories throughout the fermentation process (from 0 h to post-refrigeration, Pr) for control (in blue) and lupin-enriched (in red) yogurts. From (a) to (l): acetate,

benzoate, citrate, formate, fumarate, hippurate, lactate, malonate, orotate, 2-oxoglutarate, pyruvate, and succinate. Dashed boxes for measurements in post-refrigeration identify statistically significant differences between control and lupin-enriched yogurts. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; and \*\*\*  $p < 0.001$  express statistical relevance between a time point and the preceding one.

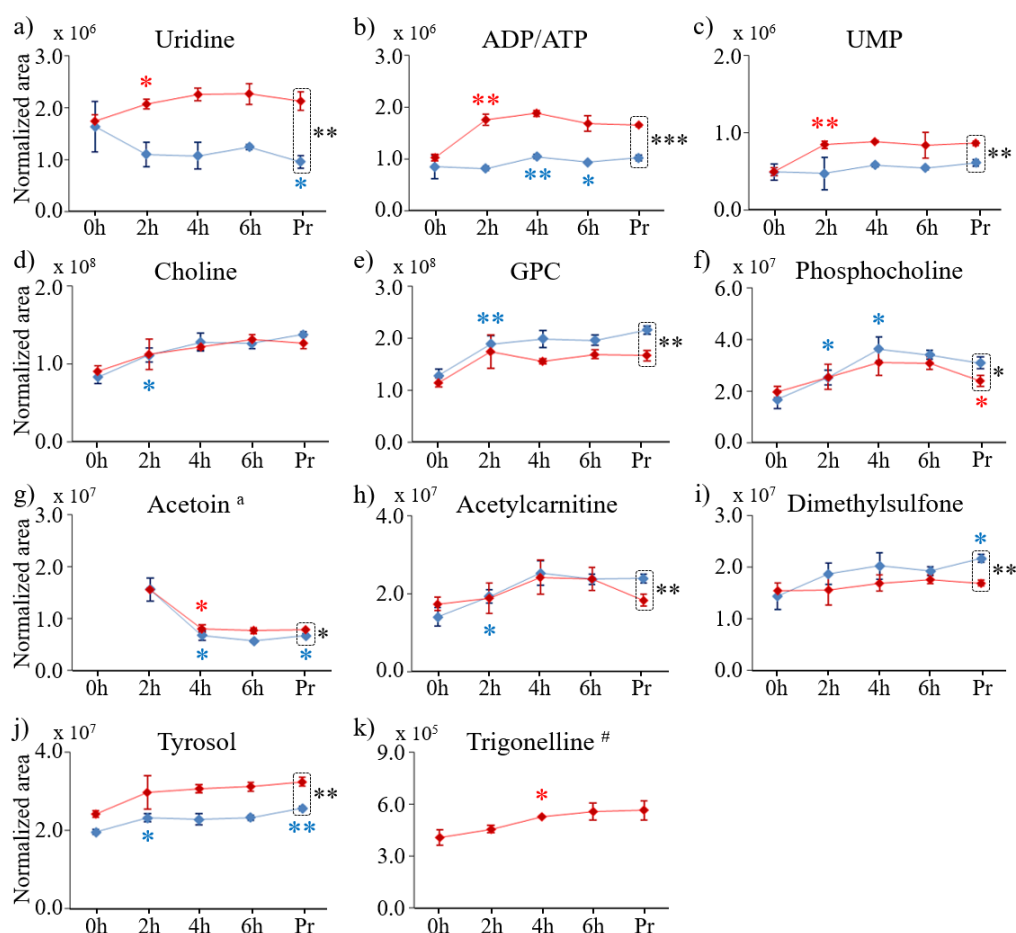
The levels of amino acids (and their derivatives) also increase from 0 to 2 h of fermentation (namely betaine, creatine, creatinine, phenylalanine and tyrosine; ES ranging from 2.1 (creatinine) to 7.9 (betaine)), with betaine subsequently returning to the lower initial levels (Figure 4.4 and Table S2). In addition, alanine, isoleucine and valine decrease up to 4 h (Figure 4.4). As observed for organic acids, amino acids are not seen to vary significantly from 4 to 6 h, whereas most amino acids increase again upon refrigeration, ranging between ES = 1.9 (betaine) and ES = 4.7 (phenylalanine) (Table S2 and Figure 4.4). Regarding sugars, a notable increase in galactose is observed at 2 h (ES = 63) (Table S2 and Figure 4.4), then increasing slightly along with lactose, although the latter showed no significance (Figure 4.4). Decreases are observed in glucose-1-phosphate (G1P) (ES = -2.2), galactose-1-phosphate (Gal1P) (ES = -2.6) and UDP-galactose (ES = 5.0) (Table S2 and Figure 4.4), although again no significant changes take place from 4 to 6 h. Upon refrigeration, a small increase in galactose is noted (ES = 2.2).



**Figure 4.4.** Amino acids and derivatives and sugar metabolites trajectories throughout the fermentation process (from 0h to post-refrigeration, Pr) for control (in blue) and lupin-enriched (in red) yogurts. From (a) to (j) amino acids and derivatives: alanine, betaine, creatine, creatinine, glutamate, isoleucine, histidine, phenylalanine, tyrosine and valine; from (k) to (q) sugars and derivatives:  $\alpha$ -glucose, glucose-1-phosphate (G1P), galactose, galactose-1-phosphate (Gal1P), lactose, *N*-acetylglucosamine, and uridine diphosphate-galactose (UDP-galactose). Dashed boxes for measurements in post-refrigeration identify statistically significant differences between control and lupin-enriched yogurts. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; and \*\*\*  $p < 0.001$  express statistical relevance between a time point and the preceding one.

Other changes during fermentation in controls include: i) significant increases in choline, glycerophosphocholine (GPC) and phosphocholine (PC), acetylcarnitine,

dimethylsulfone and tyrosol (ES ranging between 1.8, for acetylcarnitine, and 4.5, for tyrosol), ii) a qualitative decrease in uridine and iii) some variations in ADP/ATP (fluctuation) and acetoin (decrease) (Table S2 and Figure 4.5). Regarding these compounds, refrigeration is accompanied by decreased uridine (ES = -2.4) and increased acetoin, dimethylsulfone and tyrosol (ES in 2.3-3.3 range).



**Figure 4.5.** Nucleotides derivatives, choline and other metabolites trajectories throughout the fermentation process (from 0h to post-refrigeration, Pr) for control (in blue) and lupin-enriched (in red) yogurts. From (a) to (c) nucleosides and derivatives: uridine, adenosine diphosphate and/or triphosphate (ADP/ATP), and uridine monophosphate (UMP); from (d) to (f) choline metabolites: choline, glycerophosphocholine (GPC), and phosphocholine; and from (g) to (k) other metabolites: acetoin, acetylcarnitine, dimethylsulfone, tyrosol, and trigonelline. <sup>a</sup> the acetoin peak was not integrated at 0 h due to major overlap with lipid resonances; <sup>#</sup> only present in lupin-enriched samples. Dashed boxes for measurements in post-refrigeration identify statistically significant differences between control and lupin-enriched yogurts. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; and \*\*\*  $p < 0.001$  express statistical relevance between a time point and the preceding one.

#### 4.3.4 Metabolic changes during fermentation of lupin-enriched yogurt

In lupin-enriched samples, a total of 23 compounds show changes during the fermentation process, interestingly revealing a distinct behavior over time, compared to

controls (Table S3). In terms of organic acids, initial (2 h) increases in formate (ES = 5.0), fumarate (ES = 6.6) and lactate (ES = 8.4) were observed (whereas no marked increases occurred in controls), to which increases in benzoate and orotate at 4 h (ES = 3.1), and succinate at 6 h (ES = 2.2) are added. Inspection of metabolite trajectories (Figure 4.3) shows that acetate and formate levels are consistently maintained above those in controls, whereas lupin-enriched samples are less concentrated in citrate, fumarate, hippurate and lactate (confirming visual spectral observations, Figure 4.1). Broadly overlapping trajectories between controls and lupin-enriched samples are noted for benzoate, malonate, orotate (although tending to higher levels in lupin samples), 2-oxoglutarate and pyruvate (Figure 4.3). Contrary to controls, more changes take place from 4 to 6 h, with only formate (ES = -3.9) and fumarate (ES = 2.3) varying with refrigeration (Table S3).

In relation to amino acids and derivatives, the general increase observed in controls is replaced by initial (2 h) variations in both directions in a smaller set of amino acids, followed by a general uptake of alanine, betaine, isoleucine and valine at 4 h (ES between -2.9, for betaine, and -8.4, for isoleucine), and of phenylalanine at 6 h (ES = -5.3) (Table S3). No amino acid changes are induced upon refrigeration, again contrary to the general increase noted in controls (Table S3). Trajectory graphs (Figure 4.4) illustrate consistently lower levels of creatine, creatinine, phenylalanine (and to a lesser extent, isoleucine, tyrosine and valine), whereas higher levels of glutamate and histidine characterize lupin samples from 0 h. In relation to sugars, lupin-enriched samples show a much-attenuated galactose increase, compared to controls, in tandem with an increase in lactose (from 2 to 4 h, ES = 12.3) reaching a plateau of lower levels compared to controls (Figure 4.4 and Table S3). Lupin-enriched samples are characterized by lower levels of galactose, lactose (confirming visual observations) and, to lesser extents, of G1P, Gal1P and *N*-acetylglucosamine; conversely, higher stable UDP-galactose levels are found in lupin-enriched samples (Figure 4.4). Other compounds show relative increases at 2 h, such as uridine, ADP/ATP and UMP, which are kept at higher levels, compared to controls. Other significant differences include higher levels of tyrosol, presence of trigonelline (only detected in lupin samples) (Figure 4.5) and no influence of refrigeration on these compounds (leaving fumarate, formate and PC as the only varying metabolites during that final step).

Indeed, the effect of refrigeration is a significantly distinct feature of lupin samples (composition hardly changes), relatively to controls (generally increase in a large set of metabolites). Further direct comparison of the two end-products (Figures 4.3-4.5,

dashed boxes at time point Pr, and Table S4) shows that lupin-enriched yogurt differs from traditional yogurt, by higher amounts of acetate, formate, glutamate, histidine, UDP-galactose, uridine, ADP/ATP, UMP, acetoin, tyrosol, and presence of trigonelline. The same samples have relatively lower contents of citrate, fumarate, hippurate, lactate, malonate, 2-oxoglutarate, amino acids and sugars in general (except for glutamate, histidine and UDP-galactose), GPC and PC, acetylcarnitine and dimethylsulfone.

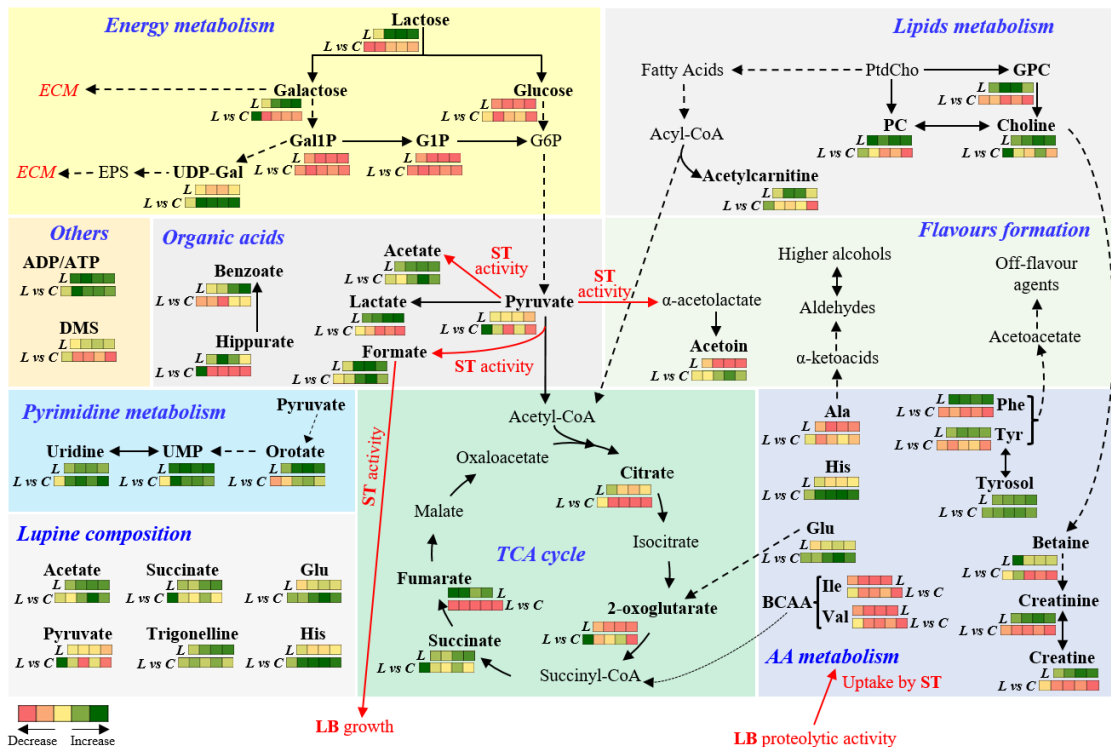
#### 4.4 Discussion

The traditional yogurt-making process involves milk fermentation promoted by symbiotic *S. thermophilus* (ST) and *L. bulgaricus* (LB) bacterial starter cultures, known to promote the conversion of lactose into lactic acid (fermentation), hydrolysis of milk caseins into peptides and free amino acids (proteolysis), and breakdown of milk fat into free fatty acids (lipolysis) (Settachaimongkon *et al.*, 2016; Wang *et al.*, 2021). Our results show that, in the presence of lupin, the complex interplay of the above processes is significantly distinct, both in terms of metabolic dynamics and profile.

Regarding dynamics, it is interesting that control conditions involve many changes between 0 and 2 h, with almost no changes in the 4-6 h period, followed by a final set of (weaker) changes upon refrigeration. On the contrary, in the presence of lupin, metabolite changes spread over the whole 6 h period, with refrigeration having hardly any impact in the final product composition. This interesting dynamic distinction suggests that bacterial biochemistry kinetics is significantly affected (delayed) in the presence of lupin, with refrigeration not affecting end-product composition. We suggest that the apparently delayed kinetics may relate to the presence of antimicrobial compounds or metabolic inhibitors related to lupin, either present in its constitution (as is the case of trigonelline) or formed during fermentation (although the contribution of possible small differences in pH or final viable cell numbers may not be ruled out, at this stage).

In relation to biochemical profile, control conditions lead to the expected increase in lactate, resulting from the fermentation of lactose by both ST and LB cultures. The high levels of lactose remain practically unchanged (as viewed by NMR), as this disaccharide composes *ca.* 6.5% of yogurt and is expected to decrease progressively to about 4.2%, during fermentation (Toba *et al.*, 1983). Lactose is converted into glucose and galactose during fermentation (Sørensen *et al.*, 2016). It seems, however, that glucose is the main glycolysis substrate, rather than galactose (which increases), to form pyruvate and then lactate, as well as formate and acetate (Figure 4.6). Indeed, since most ST strains and LB

strains are known to be unable to ferment galactose (Sørensen *et al.*, 2016), this sugar is expected to accumulate in the yogurt matrix (Trimigno *et al.*, 2020; Sørensen *et al.*, 2016) and/or follow on to UDP-Galactose formation to support exopolysaccharide synthesis (Wang *et al.*, 2021; Sørensen *et al.*, 2016) (Figure 4.6). The slightly lower levels of glucose, intermediates G1P and Gal1P, and galactose in lupin yogurt may arise from the lower lactose content in these samples. However, none of these sugar variations were reflected in changes in total carbohydrate content (lupin-enriched yogurt, 9.3 g/100 g; and control yogurt, 8.9 g/100g) (Vieira *et al.*, 2022), which may be determined mainly by polysaccharides. As identical quantities/types of milk were used, the lower lactose content in lupin samples could tentatively be explained by a higher retention of the disaccharide within the lupin-derived polysaccharides matrices (fiber) and/or protein moieties present in lupin (this would also be consistent with the reported higher viscosity of lupin-yogurt (Vieira *et al.*, 2022)). The increase in lactose levels observed from 2 to 4 h in lupin yogurt may indicate the breakdown of those polysaccharides or protein matrixes, coincidentally with that of lipids (observed to occur from 2 to 4 h), resulting in higher availability of lactose compared to 0 h (but still lower than control levels). We also propose that the lower galactose levels in lupin yogurt may also partially arise from a concomitant enhanced production of UDP-galactose (higher levels in lupin yogurt).



**Figure 4.6.** Putative metabolic relationships between altered metabolites in lupin-enriched samples ( $p < 0.05$ ), as a function of fermentation time: i) top coloured bar expresses variations between consecutive timepoints (or trajectories), 4 squares for 0-2 h, 2-4 h, 4-6 h, 6 h-Pr); and bottom coloured bar expresses differences between L and C samples at each timepoint, 5 squares for 0, 2, 4, 6 h and Pr). Box colour indicates variation direction: red, decrease; green, increased. Metabolites detected by NMR are shown in bold. Red arrows show pathways specifically influenced by ST or LB activity. Abbreviations: AA, amino acid; BCCA, branched chain amino acids; CoA, coenzyme A; DMS, dimethylsulfone; ECM, extracellular medium; G1P, glucose-1-phosphate; Gal1P, galactose-1-phosphate; G6P, glucose-6-phosphate; LB, *Lactobacillus delbrueckii* subsp. *bulgaricus*; PtdCho, phosphatidylcholine; UDP-Gal, uridine diphosphate-galactose; ST, *Streptococcus thermophilus*, TCA, tricarboxylic acid; three-letter code used for amino acids; other abbreviations as defined in the caption of Figure 4.1.

Besides conversion to lactate, pyruvate can be further converted into citrate, and particularly due to ST action, into acetate, formate, acetaldehyde, acetoin and diacetyl (Trimigno *et al.*, 2020). In lupin yogurt, acetate and formate synthesis seems to be favored in relation to citrate and fumarate synthesis. We suggest that the ST/LB mechanisms leading to citrate and fumarate formation may be inhibited by lupin components, thus leading to significant depletion of those acids. As fumarate and malate (not detected in this work) are known to be metabolized into succinate by LB strains (Yamamoto *et al.*, 2021), it is interesting to note that despite the lower levels of fumarate in lupin yogurt, succinate levels remain similar to controls, probably supported by an alternative source. Conversely, formate levels are much enhanced in lupin yogurt. Formate can be produced from pyruvate through the action of ST, whereas LB cannot produce formate and, instead, employs it for growth (Yamamoto *et al.*, 2021). This is the result of ST/LB proto-cooperation, which leads to carbon dioxide, folate, pyruvate and formate formation through ST, and release of peptides and amino acids through the proteolytic action of LB (Trimigno *et al.*, 2020; Yamamoto *et al.*, 2021) (Figure 4.6). The higher formate levels in lupin yogurt may indicate an enhanced ST action, compared to that of LB. This would be consistent with the more modest increases in creatine, creatinine, phenylalanine and tyrosine, until 6 h, after which a formate decrease during refrigeration may indicate that LB is using this organic acid faster than ST can produce it. Still regarding organic acids, benzoate increases with fermentation in both yogurt types, although involving slightly lower levels in lupin yogurt, up to 4 h. Benzoate is an antimicrobial and antifungal agent, which is produced by lactic bacteria during fermentation, and which plays a critical role in the stability of fermented milk qualities during storage (Huang *et al.*, 2022). As hippurate is a precursor of benzoate, the lower hippurate levels in lupin yogurt (perhaps again due to its retention within heavily hydrogen-bonded polysaccharide matrixes) may

explain the slightly lower benzoate levels, although not compromising benzoate content in the end-product. Orotate is an intermediate in pyrimidine biosynthesis (Lemos *et al.*, 2022), and its increased levels in lupin yogurt are likely to relate to the enhanced levels of uridine (although slightly decreased with refrigeration), UMP and UDP-Galactose.

Regarding amino acids and derivatives, LB proteolytic action in controls may be particularly responsible for the noted increases in betaine (from 0 to 2 h), creatine, creatinine, phenylalanine and tyrosine. Indeed, it has been reported that increased levels of aromatic amino acids may be related to protein degradation (Wang *et al.*, 2021). The general progression of amino acid levels is similar between controls and lupin yogurts, although the latter contain lower relative levels of creatine, creatinine, phenylalanine (and to a lesser extent, isoleucine, tyrosine and valine). This may corroborate decreased LB proteolytic action, as suggested above. In addition, refrigeration seems to enhance amino acid levels in controls but not in lupin-containing samples, an interesting observation which will require further investigation. We further suggest that lupin-enriched yogurts are enriched in glutamate and histidine from 0 h due to their presence in lupin (such amino acids show, however, no dependence on fermentation time). In fact, glutamate has been reported as the most abundant amino acid in lupin (Sujak *et al.*, 2006). Finally, the early alanine, isoleucine and valine decreases noted in both yogurt types may relate to their anaplerotic use feeding the tricarboxylic acid cycle (TCA) for ATP production (Mann *et al.*, 2021), particularly by ST bacteria (Zourari *et al.*, 1992). Alanine is also known to be used in peptidoglycans synthesis, whereas branched chain amino acids such as isoleucine and valine are important substrates for both strains and, thus, expected to be up taken early in fermentation. In addition, amino acid conversion to flavor-compounds may be occurring, through deamidation and decarboxylation to produce  $\alpha$ -ketoacids. These can be further metabolized to alcohols, esters and acids (Xiao *et al.*, 2023). Such end-products were, however, not detected in the NMR spectra, perhaps due to their low concentrations.

Similar behavior was noted for the two yogurt types regarding the remaining compounds detected. Namely, similar behavior is seen for choline compounds (increasing tendency, particularly at initial fermentation times), with higher levels of choline having been related to disruption of casein micelles (Maher *et al.*, 2013) and storage conditions (Lemos *et al.*, 2022). The decrease in acetoin, equivalent for both yogurt types, may reflect the similar change for valine. Indeed, a study of ST fermentation using l-valine as a supplement in soymilk whey showed that enhanced acetoin production from  $\alpha$ -acetolactate occurred, rather than through nonenzymatic oxidation from  $\alpha$ -acetolactate to

diacetyl (Kaneko *et al.*, 2014). Other similar features for both types of yogurts include acetylcarnitine and dimethylsulfone (both slightly increased), which respectively should reflect fatty acid transport and oxidation (Longo *et al.*, 2016) (as shown to occur in the 0-4 h period of fermentation) and the catabolism of sulfur amino acids, in particular methionine (Villeneuve *et al.*, 2013). Finally, our results have confirmed the alkaloid hormone trigonelline as a recognized indicator of lupin addition (Martínez-Villaluenga *et al.*, 2006), a metabolite which regulates plant cell cycle functions, nodulation and oxidative stress, thus generally supporting plant survival and growth (Gupta *et al.*, 2021). Interestingly, trigonelline is seen to increase slightly at 4 h, probably due to initial hydrophilic retention within biopolymeric matrixes.

In summary, for the first time to our knowledge, untargeted metabolomics (using NMR) revealed detailed compositional changes taking place during yogurt fermentation in the presence of added lupin flour, compared to traditional control conditions. Our results unveiled that the use of lupin flour significantly changes the complex interplay of ongoing biochemical processes, as reflected in terms of the evolution of metabolic profile over time. Specifically, in the presence of lupin, metabolic changes seem to reflect an apparent delayed microbial action (with refrigeration not impacting on end-product composition), accompanied by significant changes in the levels of specific organic acids, amino acids, some sugars, nucleotides and choline compounds. In particular, lupin seems to favor acetate and formate synthesis, compared to that of citrate and fumarate, consistently with higher levels of *S. thermophilus* action, relatively to *L. bulgaricus*. In addition, further observations indicated that lupin-yogurt is poorer in hippurate, lactose (and lactate), galactose, glucose-1-phosphate and galactose-1-phosphate, while containing higher orotate and trigonelline (lupin marker) levels, among other differences.

This detailed compositional knowledge adds to previously reported beneficial nutritional and rheological properties of lupin-yogurt (although a slightly undesirable sensorial profile was noted), supporting additional benefits of the presence of specific bioactive metabolites and supporting possible further developments of a new lupin-enriched yogurt.

## *Supplementary Information*

**Table S1.** List of compounds and corresponding spin systems identified in 500 MHz  $^1\text{H}$  NMR spectra of the yogurts under study.

**Table S2.** Effect size (ES) values of varying metabolites ( $p < 0.05$ ) identified in control samples (traditional fermentation conditions), as a function of time.

**Table S3.** Effect size (ES) values of varying metabolites ( $p < 0.05$ ) identified in lupin-enriched samples as a function of time.

**Table S4.** Effect size (ES) values of varying metabolites ( $p < 0.05$ ) in yogurts produced with lupin-enrichment and post-refrigeration (PrL), compared to those produced under control conditions (PrC).

**Figure S1.** General workflow of the production of control and lupin-enriched yogurts and selected sampling time points for NMR metabolomics.

**Figure S2.** Spectral expansions of the aliphatic (0.8 – 2.5 ppm) and sugar (5.0 – 5.5 ppm) regions of the 500 MHz  $^1\text{H}$  NMR spectra of samples produced: a) with lupin enrichment, and b) under control conditions, as a function of fermentation time.

**Figure S3.** PCA and PLS-DA obtained with  $^1\text{H}$  NMR spectra of: a) control samples (blue, C); and b) lupin-enriched samples (red, L); and c) LV1 loadings plot (colored according to VIP) corresponding to the PLS-DA model in (b).

**Table S1.** List of compounds and corresponding spin systems identified in 500 MHz <sup>1</sup>H NMR spectra of the yogurts under study. The metabolite peaks used for integration are shown in bold and underlined. s, singlet; d, doublet; dd, double of doublets; t, triplet; q, quartet; m, multiplet. <sup>a</sup> FooDB compounds ID code based on [www.foodb.ca](http://www.foodb.ca) (accessed in 10-05-2023). <sup>b</sup> HMDB metabolites code based on (22); <sup>c</sup> no integration due to major overlap with other resonances; <sup>d</sup> metabolite only present in lupin-rich samples.

	Compound	$\delta$ <sup>1</sup> H in ppm (multiplicity, assignment)	FooDB ID <sup>a</sup>	HMDB ID <sup>b</sup>
Organic acids	Acetate	<b>1.92</b> (s)	FDB008299	HMDB0000042
	Benzoate	<b>7.49</b> (t); 7.55 (t); 7.88 (d)	FDB008739	HMDB0001870
	Citrate	<b>2.54</b> (d); 2.69 (d)	FDB012586	HMDB0000094
	Formate	<b>8.46</b> (s)	FDB012804	HMDB0000142
	Fumarate	<b>6.52</b> (s)	FDB003291	HMDB0000134
	Hippurate	7.56 (t); 7.64 (t); <b>7.84</b> (d)	FDB001819	HMDB0000714
	Lactate	<b>1.33</b> (d); 4.12 (q)	FDB003292	HMDB0000190
	Malonate	<b>3.13</b> (s)	FDB008117	HMDB0000691
	Orotate	<b>6.18</b> (s)	FDB031072	HMDB0000226
	Pyruvate	<b>2.37</b> (s)	FDB008293	HMDB0000243
	Succinate	<b>2.41</b> (s)	FDB001931	HMDB0000254
	2-oxoglutarate	<b>2.44</b> (s); 3.01 (t)	FDB003361	HMDB0000208
	Amino acids & derivatives	Alanine	<b>1.49</b> (d); 3.78 (q)	FDB000556
Betaine		<b>3.27</b> (s); 3.89 (s)	FDB009020	HMDB0000043
Creatine		<b>3.04</b> (s); 3.92 (s)	FDB005403	HMDB0000064
Creatinine		<b>3.05</b> (s); 4.05 (s)	FDB021814	HMDB0000562
Glutamate		2.04 (m); 2.11 (m); <b>2.35</b> (m); 3.77 (t)	FDB012535	HMDB0000148
Isoleucine		0.94 (t); <b>1.01</b> (d); 1.29 (m); 1.48 (m); 1.99 (m); 3.67 (d)	FDB012397	HMDB0000172
Leucine <sup>c</sup>		0.96 (t); 1.71 (m); 1.74 (m); 3.74 (t)	FDB001946	HMDB0000687
Histidine		3.37 (dd); 4.12 (dd); <b>7.10</b> (s); 7.88 (s)	FDB011856	HMDB0000177
Methionine <sup>c</sup>		2.14 (m); 2.65 (t); 3.89 (dd)	FDB012683	HMDB0000696
Proline <sup>c</sup>		2.01 (m); 2.06 (m); 2.34 (m); 3.35 (t); 3.41 (t); 4.23 (t)	FDB000570	HMDB0000162
Phenylalanine		3.19 (m); 4.00 (m); 7.34 (m); 7.40 (m); <b>7.43</b> (m)	FDB004940	HMDB0000159
Tyrosine		3.06 (dd); 3.19 (dd); 3.96 (dd); <b>6.90</b> (d); 7.20 (d)	FDB000446	HMDB0000158
Valine		0.99 (d); <b>1.05</b> (d); 2.28 (m); 3.62 (d)	FDB012740	HMDB0000883
Sugars & derivatives	$\alpha$ -glucose	3.41 (m); 3.54 (dd); 3.72 (t); 3.77 (t); 3.83 (m); <b>5.21</b> (d)	FDB011829	HMDB0003345
	$\beta$ -glucose <sup>c</sup>	3.24 (dd); 3.41 (m); 3.47 (m); 3.50 (t); 3.73 (dd); <b>4.65</b> (d)	FDB01182	HMDB0003345
	Glucose-1-phosphate	3.37 (t); 3.46 (m); 3.48 (m); 3.70 (d); 3.89 (m); <b>5.50</b> (dd)	FDB031264	HMDB0001586
	Galactose	3.93 (d); 3.99 (d); 4.09 (dd); <b>4.59</b> (d); 5.27 (d)	FDB012703	HMDB0000143
	Galactose-1-phosphate	3.72 (d); 4.02 (dd); 4.12 (t); <b>5.35</b> (dd)	FDB031262	HMDB0000645
	Lactose	3.55 (dd); 3.60 (dd); 3.91 (d); 3.94 (d); <b>4.46</b> (d); 4.68 (d); 5.24 (d)	FDB001145	HMDB0000186
	<i>N</i> -acetylglucosamine	<b>2.06</b> (s); 5.19 (d)	FDB008032	HMDB0000215
	UDP-galactose	6.00 (m); <b>7.94</b> (d)	FDB028928	HMDB0012305
	Nucleosides & derivatives	Uridine	4.23 (dd); 4.38 (dd); 5.90 (d); <b>5.93</b> (t); 7.83 (d)	FDB007411
ADP and/or ATP		4.28 (q); 4.44 (dd); 6.13(d); <b>8.28</b> (s); 8.62 (s)	FDB021817 FDB030683	HMDB0001341 HMDB0000538
UMP		4.01 (m); 4.37 (t); 4.43 (t); 5.98 (m); <b>8.12</b> (d)	FDB031248	HMDB0000288
Choline metabolites	Choline	<b>3.20</b> (s); 3.50 (m); 4.06 (m)	FDB000710	HMDB0000097
	GPC	<b>3.23</b> (s); 3.65 (m); 4.33 (m)	FDB021802	HMDB0000086
	Phosphocholine	<b>3.22</b> (s); 4.15 (m)	FDB022692	HMDB0001565
Othermetabolites	Acetoin	1.38 (d); <b>2.44</b> (s); 4.41 (q)	FDB011799	HMDB0003243
	Acetylcarbitine	2.13 (s); 2.50 (dd); 2.64 (dd); <b>3.20</b> (s); 3.61 (d); 3.8 (dd); 5.60 (q)	FDB021904	HMDB0000201
	Dimethyl sulfone	<b>3.16</b> (s)	FDB006725	HMDB0004983
	Tyrosol	<b>6.86</b> (d); 7.17 (d)	FDB031233	HMDB0004284
	Trigonelline <sup>d</sup>	4.43 (s); 8.09 (t); 8.85 (d); <b>9.13</b> (s)	FDB002237	HMDB0000875

**Table S2.** Effect size (ES) values of varying metabolites ( $p < 0.05$ ) identified in control samples (traditional fermentation conditions), as a function of time ( $n = 3$  per condition). <sup>a</sup> integrated peak within spin system; <sup>b</sup> effect size and corresponding error; <sup>c</sup> significance level 95% ( $p < 0.05$ ). \* Metabolites with  $p < 0.05$ , after correction by the Benjamin-Hochberg false discovery rate method (corrected  $p$ -values ranged between  $1.6 \times 10^{-2}$  and  $4.4 \times 10^{-2}$ ).

Metabolites		$\delta_H$ , ppm (multiplicity) <sup>a</sup>	2hC vs. 0hC		4hC vs. 2hC		6hC vs. 4hC		PrC vs. 6hC	
			ES $\pm$ error <sup>b</sup>	$p$ -value <sup>c</sup>	ES $\pm$ error <sup>b</sup>	$p$ -value <sup>c</sup>	ES $\pm$ error <sup>b</sup>	$p$ -value <sup>c</sup>	ES $\pm$ error <sup>b</sup>	$p$ -value <sup>c</sup>
Organic acids	Acetate	1.92 (s)	1.7 $\pm$ 1.6	$4.5 \times 10^{-2}$	---	---	---	---	2.4 $\pm$ 1.9	$2.3 \times 10^{-2}$
	Benzoate	7.49 (t)	---	---	3.4 $\pm$ 2.3	$6.6 \times 10^{-3}$	2.6 $\pm$ 1.9	$4.2 \times 10^{-2}$	7.1 $\pm$ 4.2	$8.6 \times 10^{-4}$ *
	Citrate	2.54 (d)	3.5 $\pm$ 2.4	$4.8 \times 10^{-3}$ *	---	---	---	---	---	---
	Formate	8.46 (s)	4.5 $\pm$ 2.9	$7.2 \times 10^{-3}$ *	6.9 $\pm$ 4.1	$8.4 \times 10^{-3}$	---	---	2.3 $\pm$ 1.8	$3.1 \times 10^{-2}$
	Fumarate	6.52 (s)	9.6 $\pm$ 5.6	$1.3 \times 10^{-4}$ *	1.9 $\pm$ 1.7	$5.0 \times 10^{-2}$	---	---	3.3 $\pm$ 2.3	$1.2 \times 10^{-2}$
	Hippurate	7.84 (d)	11.2 $\pm$ 6.5	$3.9 \times 10^{-2}$	---	---	---	---	---	---
	Lactate	1.33 (d)	33.6 $\pm$ 19.1	$1.0 \times 10^{-2}$ *	2.9 $\pm$ 2.1	$1.1 \times 10^{-2}$	---	---	3.3 $\pm$ 2.3	$1.2 \times 10^{-2}$
	Orotate	6.18 (s)	5.8 $\pm$ 3.5	$7.9 \times 10^{-4}$ *	---	---	---	---	2.9 $\pm$ 2.1	$4.2 \times 10^{-2}$
	Pyruvate	2.37 (s)	2.6 $\pm$ 1.9	$1.3 \times 10^{-2}$ *	---	---	---	---	---	---
	Succinate	2.41 (s)	3.9 $\pm$ 2.6	$1.8 \times 10^{-2}$ *	---	---	---	---	3.8 $\pm$ 2.5	$9.2 \times 10^{-3}$
	2-oxoglutarate	2.44 (s)	---	---	---	---	---	---	2.5 $\pm$ 1.9	$2.1 \times 10^{-2}$
Amino acids & derivatives	Alanine	1.49 (d)	---	---	-2.3 $\pm$ 1.8	$3.2 \times 10^{-2}$	---	---	---	---
	Betaine	3.27 (s)	7.9 $\pm$ 4.6	$6.3 \times 10^{-3}$ *	-2.2 $\pm$ 1.8	$3.6 \times 10^{-2}$	---	---	1.9 $\pm$ 1.7	$4.6 \times 10^{-2}$
	Creatine	3.04 (s)	2.3 $\pm$ 1.8	$2.1 \times 10^{-2}$	---	---	---	---	2.0 $\pm$ 1.7	$4.7 \times 10^{-2}$
	Creatinine	3.05 (s)	2.1 $\pm$ 1.8	$2.4 \times 10^{-2}$	---	---	---	---	2.1 $\pm$ 1.7	$3.3 \times 10^{-2}$
	Glutamate	2.35 (m)	---	---	---	---	---	---	2.2 $\pm$ 1.8	$2.9 \times 10^{-2}$
	Isoleucine	1.01 (d)	---	---	-3.0 $\pm$ 2.1	$2.3 \times 10^{-2}$	---	---	2.1 $\pm$ 1.7	$4.9 \times 10^{-2}$
	Phenylalanine	7.43 (m)	2.9 $\pm$ 2.1	$1.1 \times 10^{-2}$ *	---	---	---	---	4.7 $\pm$ 3.0	$3.7 \times 10^{-3}$
	Tyrosine	6.90 (d)	5.6 $\pm$ 3.4	$1.1 \times 10^{-3}$ *	---	---	---	---	2.4 $\pm$ 1.9	$2.2 \times 10^{-2}$
Sugars & derivatives	Valine	1.05 (d)	---	---	-2.2 $\pm$ 1.8	$4.1 \times 10^{-2}$	---	---	---	---
	Glucose-1-phosphate	5.50 (dd)	---	---	-2.2 $\pm$ 1.8	$3.4 \times 10^{-2}$	---	---	---	---
	Galactose	4.59 (d)	63.2 $\pm$ 35.8	$1.5 \times 10^{-2}$ *	2.0 $\pm$ 1.7	$3.6 \times 10^{-2}$	---	---	2.2 $\pm$ 1.8	$2.9 \times 10^{-2}$
	Galactose-1-phosphate	5.35 (dd)	---	---	-2.6 $\pm$ 1.9	$4.8 \times 10^{-2}$	---	---	---	---
Nucleotides & derivatives	UDP-galactose	7.94 (d)	-5.0 $\pm$ 3.1	$5.8 \times 10^{-3}$ *	---	---	---	---	---	---
	Uridine	5.93 (t)	---	---	---	---	---	---	-2.4 $\pm$ 1.9	$4.5 \times 10^{-2}$
Choline metabolites	ADP and/or ATP	8.28 (s)	---	---	4.0 $\pm$ 2.6	$3.7 \times 10^{-3}$	-2.6 $\pm$ 1.9	$4.7 \times 10^{-2}$	---	---
	Choline	3.20 (s)	2.7 $\pm$ 2.0	$1.7 \times 10^{-2}$ *	---	---	---	---	---	---
	GPC	3.23 (s)	3.8 $\pm$ 2.5	$8.6 \times 10^{-3}$ *	---	---	---	---	---	---
Other metabolites	Phosphocholine	3.22 (s)	2.0 $\pm$ 1.7	$2.9 \times 10^{-2}$	2.3 $\pm$ 1.8	$3.2 \times 10^{-2}$	---	---	---	---
	Acetoin	2.44 (d)	---	---	-4.1 $\pm$ 2.7	$1.1 \times 10^{-2}$	---	---	2.5 $\pm$ 1.9	$2.0 \times 10^{-2}$
	Acetylcarnitine	3.20 (s)	1.8 $\pm$ 1.6	$4.1 \times 10^{-2}$	---	---	---	---	---	---
	Dimethylsulfone	3.16 (s)	---	---	---	---	---	---	2.3 $\pm$ 1.8	$2.4 \times 10^{-2}$
	Tyrosol	6.86 (d)	4.5 $\pm$ 2.8	$1.3 \times 10^{-2}$ *	---	---	---	---	3.3 $\pm$ 2.3	$7.3 \times 10^{-3}$

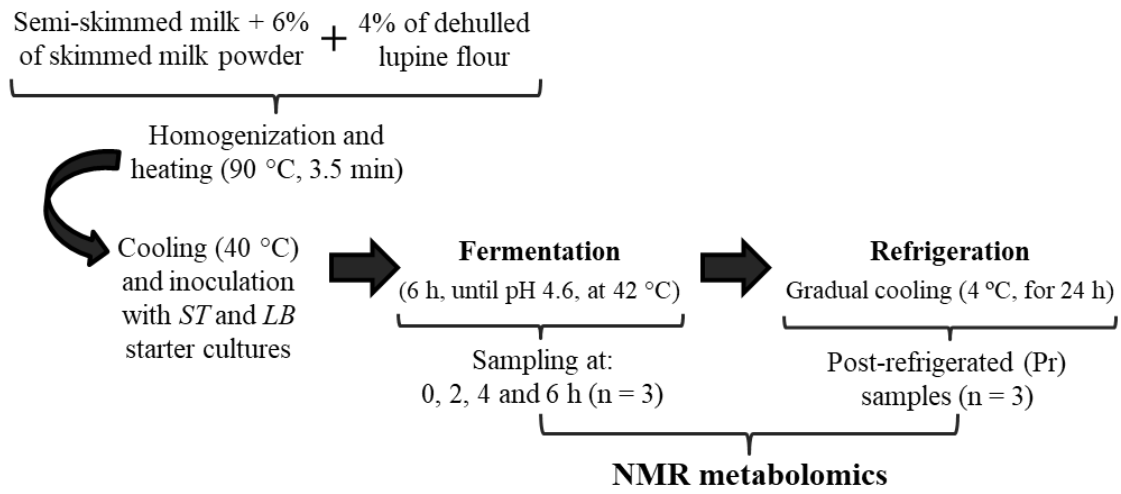
**Table S3.** Effect size (ES) values of varying metabolites ( $p < 0.05$ ) identified in lupin-enriched samples as a function of time ( $n = 3$  per condition). <sup>a</sup> integrated peak within spin system; <sup>b</sup> effect size and corresponding error; <sup>c</sup> significance level 95% ( $p < 0.05$ ). \* Metabolites with  $p < 0.05$ , after correction by the Benjamin-Hochberg false discovery rate method (corrected  $p$ -values ranged between  $3.3 \times 10^{-3}$  and  $4.2 \times 10^{-2}$ ).

Metabolites		$\delta_H$ , ppm (multiplicity) <sup>a</sup>	2hL vs. 0hL		4hL vs. 2hL		6hL vs. 4hL		PrL vs. 6hL	
			ES $\pm$ error <sup>b</sup>	$p$ -value <sup>c</sup>	ES $\pm$ error <sup>b</sup>	$p$ -value <sup>c</sup>	ES $\pm$ error <sup>b</sup>	$p$ -value <sup>c</sup>	ES $\pm$ error <sup>b</sup>	$p$ -value <sup>c</sup>
Organic acids	Benzoate	7.49 (t)	---	---	3.1 $\pm$ 2.2	1.8 $\times 10^{-2}$	4.3 $\pm$ 2.8	2.0 $\times 10^{-2}$	---	---
	Formate	8.46 (s)	5.0 $\pm$ 3.1	6.3 $\times 10^{-3}$ *	6.1 $\pm$ 3.7	8.6 $\times 10^{-3}$	---	---	-3.9 $\pm$ 2.6	6.5 $\times 10^{-3}$
	Fumarate	6.52 (s)	8.4 $\pm$ 4.9	2.4 $\times 10^{-3}$ *	---	---	-7.8 $\pm$ 4.6	3.0 $\times 10^{-4}$ *	2.3 $\pm$ 1.8	3.2 $\times 10^{-2}$
	Hippurate	7.84 (d)	---	---	---	---	---	---	---	---
	Lactate	1.33 (d)	6.6 $\pm$ 4.0	8.5 $\times 10^{-3}$ *	---	---	3.7 $\pm$ 2.4	8.8 $\times 10^{-3}$	---	---
	Orotate	6.18 (s)	---	---	2.4 $\pm$ 1.9	3.3 $\times 10^{-2}$	---	---	---	---
	Succinate	2.41 (s)	---	---	---	---	2.2 $\pm$ 1.8	2.8 $\times 10^{-2}$	---	---
Amino acids & derivatives	Alanine	1.49 (d)	-3.9 $\pm$ 2.6	1.4 $\times 10^{-2}$	-4.4 $\pm$ 2.8	1.4 $\times 10^{-2}$	---	---	---	---
	Betaine	3.27 (s)	3.4 $\pm$ 2.3	3.1 $\times 10^{-2}$	-2.9 $\pm$ 2.1	4.4 $\times 10^{-2}$	---	---	---	---
	Isoleucine	1.01 (d)	-9.0 $\pm$ 5.2	1.8 $\times 10^{-4}$ *	-8.4 $\pm$ 4.9	4.4 $\times 10^{-4}$ *	---	---	---	---
	Phenylalanine	7.43 (m)	2.9 $\pm$ 2.1	4.3 $\times 10^{-2}$	---	---	-5.3 $\pm$ 3.3	1.0 $\times 10^{-2}$	---	---
	Valine	1.05 (d)	-10.2 $\pm$ 5.9	9.8 $\times 10^{-5}$ *	-8.8 $\pm$ 5.1	1.8 $\times 10^{-3}$ *	---	---	---	---
Sugars & derivatives	$\alpha$ -glucose	5.21 (d)	-2.7 $\pm$ 2.0	1.5 $\times 10^{-2}$	---	---	---	---	---	---
	Glucose-1-phosphate	5.50 (dd)	2.8 $\pm$ 2.0	1.7 $\times 10^{-2}$	---	---	---	---	---	---
	Galactose	4.59 (d)	---	---	8.6 $\pm$ 5.0	2.1 $\times 10^{-4}$ *	2.7 $\pm$ 2.0	1.5 $\times 10^{-2}$	---	---
	Galactose-1-phosphate	5.35 (dd)	-3.1 $\pm$ 2.2	2.0 $\times 10^{-2}$	---	---	---	---	---	---
	Lactose	4.46 (d)	---	---	12.3 $\pm$ 7.1	7.6 $\times 10^{-5}$ *	---	---	---	---
Nucleotides & derivatives	Uridine	5.93 (t)	2.4 $\pm$ 1.9	2.5 $\times 10^{-2}$	---	---	---	---	---	---
	ADP and/or ATP	8.28 (s)	6.7 $\pm$ 4.0	1.4 $\times 10^{-3}$ *	---	---	---	---	---	---
	UMP	8.12 (d)	5.3 $\pm$ 3.3	1.3 $\times 10^{-3}$ *	---	---	---	---	---	---
Choline metabolites	Phosphocholine	3.22 (s)	---	---	---	---	---	---	-2.4 $\pm$ 1.9	2.1 $\times 10^{-2}$
Other metabolites	Acetoin	2.44 (d)	---	---	-10.8 $\pm$ 6.2	1.5 $\times 10^{-2}$ *	---	---	---	---
	Trigonelline	9.13 (d)	---	---	3.7 $\pm$ 2.4	2.2 $\times 10^{-2}$	---	---	---	---

**Table S4.** Effect size (ES) values of varying metabolites ( $p < 0.05$ ) in yogurts produced with lupin-enrichment and post-refrigeration (PrL), compared to those produced under control conditions (PrC) ( $n = 3$  per condition) <sup>a</sup> integrated peak within spin system; <sup>b</sup> effect size and corresponding error; <sup>c</sup> significance level 95% ( $p < 0.05$ ). \* Metabolite variations maintaining  $p < 0.05$  upon correction by the Benjamin-Hochberg false discovery rate method (corrected  $p$ -values ranged between  $3.1 \times 10^{-4}$  and  $4.4 \times 10^{-2}$ ).

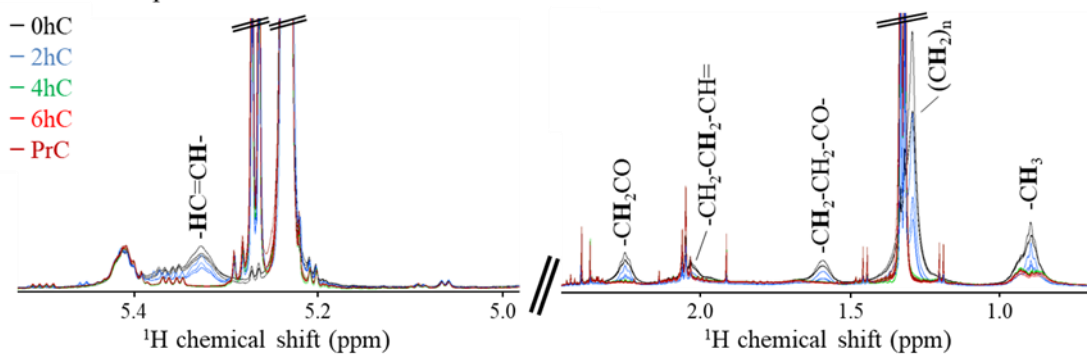
	Metabolites	$\delta^1\text{H}$ in ppm (multiplicity) <sup>a</sup>	PrL vs. PrC	
			ES $\pm$ Error <sup>b</sup>	$p$ -value <sup>c</sup>
Organic acids	Acetate	1.92 (s)	$6.2 \pm 3.7$	$3.7 \times 10^{-3}$ *
	Citrate	2.54 (d)	$-12.5 \pm 7.2$	$1.4 \times 10^{-4}$ *
	Formate	8.46 (s)	$6.7 \pm 4.0$	$1.7 \times 10^{-3}$ *
	Fumarate	6.52 (s)	$-21.1 \pm 12.0$	$7.0 \times 10^{-6}$ *
	Hippurate	7.84 (d)	$-10.2 \pm 5.9$	$3.9 \times 10^{-3}$ *
	Lactate	1.33 (d)	$-7.6 \pm 4.5$	$2.1 \times 10^{-3}$ *
	Malonate	3.13 (s)	$-3.0 \pm 2.1$	$3.6 \times 10^{-2}$ *
	2-oxoglutarate	2.44 (s)	$-2.5 \pm 1.9$	$3.6 \times 10^{-2}$ *
Amino acids and derivatives	Betaine	3.27 (s)	$-3.8 \pm 2.5$	$4.4 \times 10^{-3}$ *
	Creatine	3.04 (s)	$-7.6 \pm 4.5$	$4.8 \times 10^{-4}$ *
	Creatinine	3.05 (s)	$-6.8 \pm 4.1$	$5.5 \times 10^{-4}$ *
	Glutamate	2.35 (m)	$8.5 \pm 5.0$	$6.6 \times 10^{-4}$ *
	Isoleucine	1.01 (d)	$-2.6 \pm 1.9$	$2.1 \times 10^{-2}$ *
	Histidine	7.10 (s)	$16 \pm 9.1$	$1.7 \times 10^{-4}$ *
	Phenylalanine	7.43 (m)	$-10.0 \pm 5.8$	$4.8 \times 10^{-4}$ *
	Tyrosine	6.90 (d)	$-5.4 \pm 3.3$	$3.1 \times 10^{-3}$ *
Sugar and derivatives	Valine	1.05 (d)	$-2.8 \pm 2.0$	$1.4 \times 10^{-2}$ *
	$\alpha$ -glucose	5.21 (d)	$-5.2 \pm 3.2$	$7.0 \times 10^{-3}$ *
	Glucose-1-phosphate	5.50 (dd)	$-3.4 \pm 2.3$	$2.5 \times 10^{-2}$ *
	Galactose	4.59 (d)	$-8.3 \pm 4.9$	$5.6 \times 10^{-4}$ *
	Galactose-1-phosphate	5.35 (dd)	$-2.9 \pm 2.1$	$1.7 \times 10^{-2}$ *
	Lactose	4.46 (d)	$-6.1 \pm 3.7$	$1.3 \times 10^{-3}$ *
	<i>N</i> -acetylglucosamine	2.06 (s)	$-5.3 \pm 3.3$	$1.3 \times 10^{-3}$ *
Nucleosides derivatives	UDP-galactose	7.94 (d)	$14.6 \pm 8.4$	$3.3 \times 10^{-5}$ *
	Uridine	5.93 (t)	$6.1 \pm 3.7$	$1.3 \times 10^{-3}$ *
	ADP/ATP	8.28 (s)	$10.4 \pm 6.0$	$3.5 \times 10^{-4}$ *
	UMP	8.12 (d)	$5.7 \pm 3.5$	$1.8 \times 10^{-3}$ *
	GPC	3.23 (s)	$-4.3 \pm 2.8$	$3.6 \times 10^{-3}$ *
Other metabolites	Phosphocholine	3.22 (s)	$-2.5 \pm 1.9$	$2.0 \times 10^{-2}$ *
	Acetoin	2.44 (d)	$3.6 \pm 2.4$	$3.0 \times 10^{-2}$ *
	Acetylcarnitine	3.20 (s)	$-3.3 \pm 2.3$	$8.7 \times 10^{-3}$ *
	Dimethylsulfone	3.16 (s)	$-5.3 \pm 3.3$	$1.5 \times 10^{-3}$ *
	Tyrosol	6.86 (d)	$6.0 \pm 3.6$	$3.2 \times 10^{-3}$ *

**Figure S1.** General workflow of the production of control and lupin-enriched yogurts and selected sampling time points for NMR metabolomics. *ST*: *Streptococcus thermophiles*; *LB*: *Lactobacillus delbrueckii* subsp. *bulgaricus*.

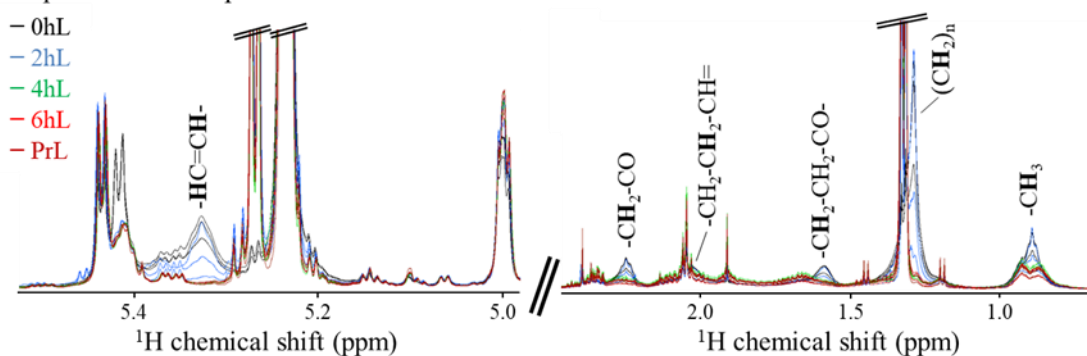


**Figure S2.** Spectral expansions of the aliphatic (0.8 – 2.5 ppm) and sugar (5.0 – 5.5 ppm) regions of the 500 MHz  $^1\text{H}$  NMR spectra of samples produced: a) with lupin enrichment, and b) under control conditions, as a function of fermentation time. Peaks assigned correspond to broad resonances corresponding to lipids mainly present in the first 2 hours of fermentation (0h, in black; and 2h, in blue) in both control and lupin-enriched samples. Lipids resonances assignment based on (28):  $-\text{CH}_3$  ( $\delta$  0.91);  $(\text{CH}_2)_n$  ( $\delta$  1.30);  $-\text{CH}_2-\text{CH}_2-\text{CO}-$  ( $\delta$  1.60);  $-\text{CH}_2-\text{CH}_2-\text{CH}=\text{}$  ( $\delta$  2.03);  $\text{CH}_2-\text{CO}$  ( $\delta$  2.26); and  $-\text{HC}=\text{CH}-$  ( $\delta$  5.33). Bold protons correspond to those for which chemical shift is indicated.

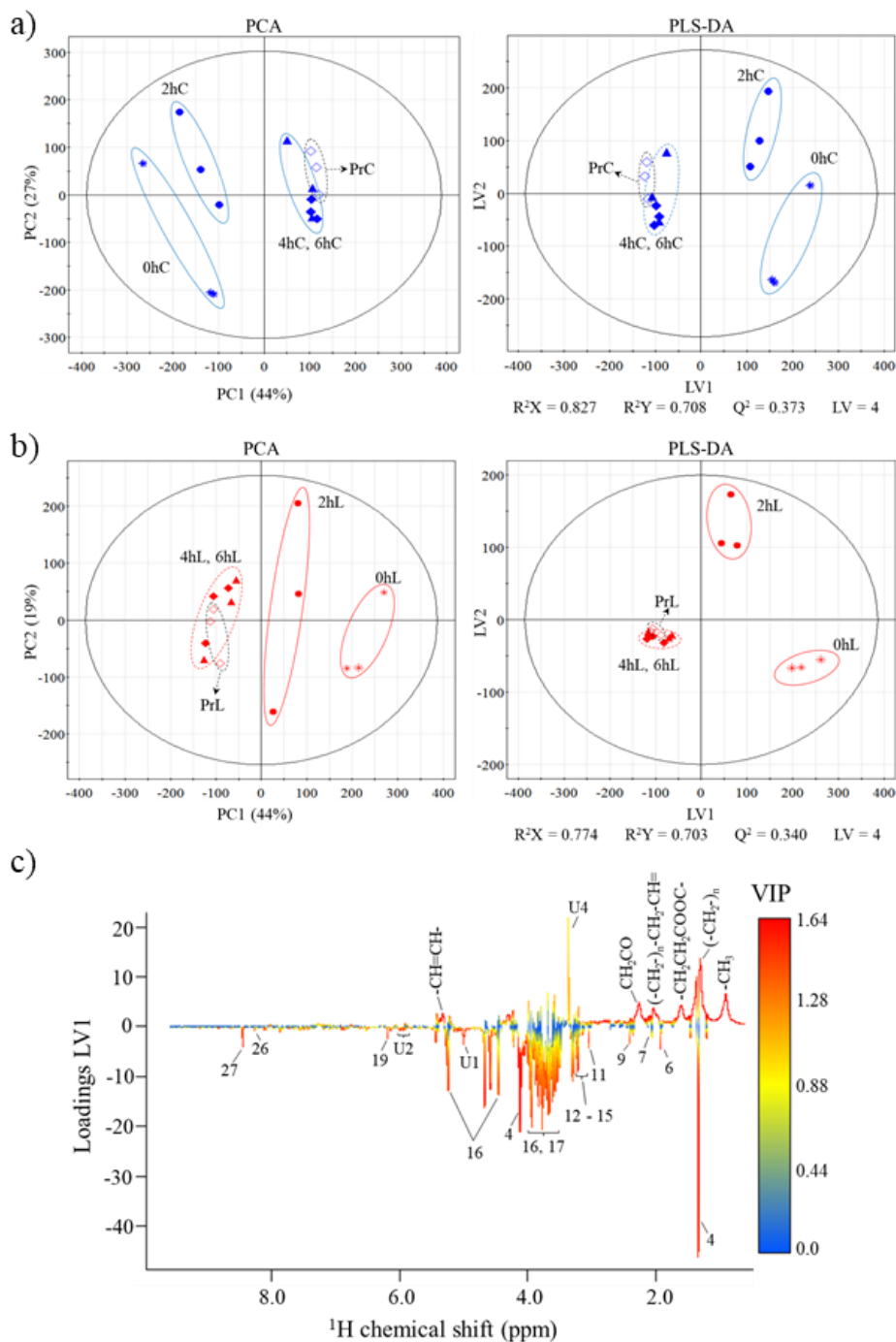
a) Control samples



b) Lupine-based samples



**Figure S3.** PCA and PLS-DA obtained with  $^1\text{H}$  NMR spectra of: a) control samples (blue, C); and b) lupin-enriched samples (red, L); and c) LV1 loadings plot (colored according to VIP) corresponding to the PLS-DA model in (b). Peak numbering in loadings corresponds to that shown in Figure 2 caption (additional peak indication for unassigned U4, singlet at  $\delta$  3.36). Symbols corresponding to fermentation time: 0h (\*); 2h (●); 4h (▲); 6h (◆); and Pr (◇). Symbols and abbreviations as shown in caption of Figure 3.



**PART III.**  
**Pulses promotion in Portuguese**  
**Kindergartens**

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## **Chapter 5. Legumes in Portuguese Kindergartens: Analysis of frequency and acceptance level, and evaluation of an educational e-book for legumes promotion**

To promote legumes in Portuguese kindergartens, an educational e-book with orientation for teachers was developed, and other possible actions were identified. An online focus group was conducted with teachers of selected kindergartens to understand their perceptions about the developed material and the consumption of pulses in these settings. The paper produced is under review:

**Vieira EDF**, Gil AM, Gomes AM, Vasconcelos MW. Leguminosas em Jardins de Infância Portugueses: Uma Análise da Frequência e Nível de Aceitação. *Acta Portuguesa de Nutrição (under review)*.

Also, part of the chapter's results were presented as a poster in the XXVII Brazilian Nutrition Congress and published at the proceedings as:

**Vieira EDF**, Gil AM, Gomes AM, Vasconcelos MW. Desenvolvimento e avaliação de um material educativo direcionado a professores da pré-escola para promover o consumo de leguminosas em Portugal. Anais do XXVII Congresso Brasileiro de Nutrição - CONBRAN 2022 - Nutrição no Ensino, na Pesquisa e na Extensão. (2022). *Revista Da Associação Brasileira De Nutrição - RASBRAN*, 13(2). 953-1160.

## **ABSTRACT**

Legumes are environmentally sustainable, nutritious, and culturally significant foods in the Mediterranean region. However, Portuguese children consume only 6% of the daily recommendations. Kindergartens play a crucial role in shaping children's eating habits, making it essential to promote the consumption of these foods in these settings. This study aimed to identify legume consumption and its associated factors in nine Portuguese kindergartens and evaluate an educative e-book according to teachers' perceptions. It is preliminary participatory research, employing both qualitative and quantitative approaches, using a structured questionnaire and an online focus group. Questionnaire data were presented in absolute numbers and frequencies, using Fisher's exact test. Data from the focus group were analyzed and interpreted using thematic content analysis. Quantitatively, it was observed that 77.8% of schools provide vegetarian meals approximately once a week. Legume grains are offered on average 3 times a week in 55.6% of schools. The acceptance level of legumes is 55.6%. The content analysis found that knowing how to cook legume grains and family involvement in food education were positive aspects for consumption. The identified barriers were meal preparation outside of school and a lack of habit of consuming legumes. According to teachers' evaluation, the material transmitted a clear message and can be used to promote the consumption of legumes. Based on these results, it is recommended that actions aimed at developing culinary skills in preparing legumes, food education with parental involvement, food preparation within the school, and the introduction of legumes in menus be prioritized in selected schools as early as possible.

**Keywords:** e-book; food supply, legume grains, kindergartens, sustainable diets.

## 5.1 Introduction

The Food and Agriculture Organization (FAO) defines sustainable diets as eating patterns that support health and well-being while staying within the limits of nature's ability to renew resources for both present and future generations (FAO and WHO, 2019). It's important to note that animal-based foods generally have a greater environmental impact per calorie or gram produced compared to plant-based foods (Springmann *et al.*, 2018). This means that shifting towards diets prioritizing plant protein over excessive animal protein is essential for reducing environmental strain (Willett *et al.*, 2019).

Legume grains have gained prominence in sustainable diets due to their low environmental impact as a protein source. By substituting animal protein with legume grains, we can alleviate pressure on natural resources like land and water while reducing greenhouse gas emissions (GHG) associated with livestock farming (Cusworth *et al.*, 2021). However, despite their significant role in sustainability, legume grain consumption remains low in most European diets (Kalogeropoulos *et al.*, 2010). Data from the Portuguese Food Balance Sheet (PFBS) indicate that legume grain consumption is nearly six times below the recommended daily intake of 80 grams of cooked legumes (INE, 2020; Rodrigues *et al.*, 2006). The average legume grain consumption among Portuguese children is about 5g daily, only 6% of the recommended amount (Rodrigues *et al.*, 2006; Lopes *et al.*, 2017). Given the environmental benefits of legume grains and the discrepancy in consumption rates, promoting their consumption among Portuguese children is crucial.

One promising approach for legume grains promotion is to modify school food environments to ensure physical, financial, political, and sociocultural access to healthy and sustainable foods (FAO and WHO, 2019; European Commission, 2021; Graça *et al.*, 2021). School meal programs provide a special opportunity to encourage healthy and sustainable eating habits because they foster strong social interactions and serve a large audience, including students, school staff, families, and community members (European Commission, 2021; Graça *et al.*, 2021). In this regard, Portugal has implemented a legislative framework governing food provision in schools, dictating which foods are promoted or offered within the school environment (Graça *et al.*, 2021). For instance, a law passed in 2017 mandates including a daily vegetarian (specifically, vegan) option in the public canteen (Brandão and Ávila, 2021), which should make legume grains the primary protein source in these menus (Lima, 2018). However, studies indicate the consumption of legume grains among Portuguese preschools has remained low (Alves, 2023; Carvalho, 2021).

The promotion of healthy and sustainable foods like legume grains should be a priority in nurseries and kindergartens since it is during the early years (3-6 years) that children begin to develop their taste preferences (Nekitsing *et al.*, 2018) and are highly receptive to behavioral influences in groups (Direção Geral de Educação, 2019). Early childhood teachers are responsible for ensuring school that meals are a learning opportunity (European Commission, 2021). Since teachers are fundamental players in food literacy and the formation of healthy habits, it is essential to know their perceptions about the consumption of legume grains at preschools and what strategies, in their opinion, can promote this food. This information may offer clues that guide actions to encourage the consumption of legume grains in Portuguese kindergartens. Also, supporting these educators with knowledge and training is sorely needed.

## **5.2 Objectives**

The objective of this study was to investigate the perceptions of early childhood teachers on legume grain consumption among a convenience sample of Portuguese kindergartens and explore the factors influencing this consumption behavior, identifying barriers or facilitators to legume grain consumption within the kindergarten environment, including menu options, peer influences, and parental attitudes. Additionally, the research aimed to develop and evaluate an educational e-book that encourages legume consumption among preschool children. Ultimately, the findings aimed to provide insights that could inform strategies for promoting healthier and more sustainable dietary habits among young children in Portugal.

## **5.3 Materials and methods**

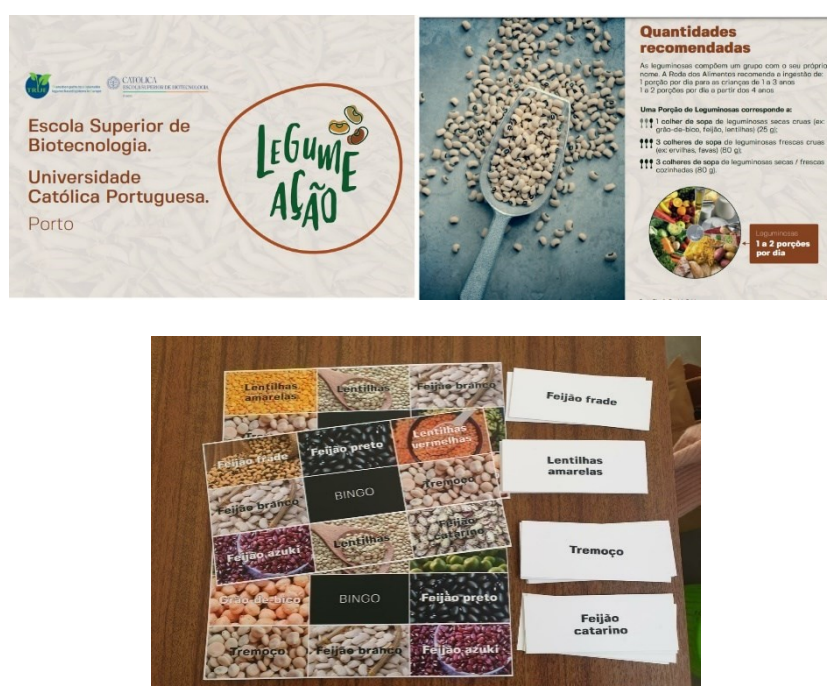
### *Material development*

The first step to in material development was a literature review carried out on official government websites, health organizations, and MEDLINE and PUBMED databases, using the following terms: “Legume”, “pulse”, “nutrition”, “preschool children”, “education intervention”, “eating behavior”, alone or in association with each other, considering the period from 2006 to 2021.

After the literature review, the e-book text and a list of suggested activities adequate for preschool children were prepared. Next, the e-book graphic project was designed and executed. In constructing the material, objective contents and a format that aroused interest,

such as colored images, visual representations, and short explanations, were considered (figure 5.1).

The e-book presents information on the importance of legume consumption in sustainable diets, its importance in meeting nutritional demands in childhood, recommendations, notions about the formation of eating habits in the preschool phase, and suggestions for activities and three educative games, which were: memory games, charades, and bingo (Figure 5.1). The activities suggested were mainly based on sensory stimuli to promote habit formation in preschoolers. Most of them are short activities (on average 10 minutes) to keep the child's attention during all the excursions.



**Figure 5.1.** Parts of the e-book elaborated for early childhood educators and games to be made with children (material in Portuguese).

### Research design

The study was preliminary participatory research in which researchers and participants discussed solutions to the problems presented. This approach is theoretically supported by the dialectical historical-structural approach, whose main objective is transforming the reality studied (Lara and Molina, 2011).

A convenience sample of nine early childhood teachers from the research team's network was invited by phone and email to participate in the study and encouraged to extend the invitation to their colleagues. After this invitation, ten educators interested in

participating in the study contacted the research team. All communication regarding the study, including its purpose and participation guidelines, was conducted via email. To be eligible, participants had to be teachers of early childhood education in Portugal and consent to participation by completing a voluntary and informed consent form. Upon agreeing to participate, individuals were directed to a link to sign the consent form and complete a structured questionnaire electronically.

The questionnaire included the following sections:

- A Questions regarding the legal status of the institution where they work (public or private)
- Whether vegetarian meals are offered on the school menu (yes or no)
- What is the frequency of legume grains on the school menu.
- If legume grains were well accepted by the children (yes or no)
- Existence of nutritional education activities (yes or no)

Besides the questionnaire, the focus group (FG) was the technical chosen to understand the perceptions and opinions of the early childhood teachers. Focus groups have been a cornerstone of qualitative research for the last 80 years (Halliday *et al.*, 2021). This methodology is particularly appropriate when the objective is to explain how people who are part of the target audience of the research consider, think, and feel about a particular subject (Schröder and Klerin, 2009), as it enables a specific number of participants (typically between 6 and 12) to exchange experiences. This method facilitates the capture of participants' expressions, beliefs, and attitudes, thereby enhancing access to information on the proposed theme (Silva *et al.*, 2013). To accommodate participants with different schedules and geographical locations, online focus groups (OFGs) were conducted using videoconferencing applications as an alternative to traditional face-to-face FG sessions (Murgado-Armenteros, *et al.*, 2012). The OFG sessions were conducted using the Zoom© videoconferencing platform.

Before the start of the focus group recordings, the participants were informed that they would be recorded, and if they withdrew from the authorization, they would have to leave the meeting. They also were informed that the recordings would be stored on a computer with access restricted to research team members. Once arrangements were made with the teachers, the online focus group (OFG) was conducted and recorded for subsequent analysis, which involved transcribing the interviews. After being transcribed, the interviews were translated into English.

Although eight of the ten educators who completed the questionnaire could participate in the focus group, one had to leave unexpectedly before engaging in the discussion, and another hadn't previously completed the necessary consent form, rendering them ineligible for participation. Consequently, the focus group proceeded with six participants in attendance.

One individual facilitated the OFG, while another took field notes to organize and guide the discussion. The focus group lasted an hour and a half and followed a predetermined script, encompassing guiding questions aligned with the study's objectives: 1) Do you think it is significant to promote legume grain consumption? How? Ex.: expanding menu options, conducting educational activities, etc. 2) From your perspective, what are the primary obstacles to legume grain consumption in schools? 3) Does the material provide relevant information on the subject and raise awareness among teachers of the importance of consuming legumes? 4) In your opinion, are the suggested activities suitable for preschoolers? 5) Are these activities capable of stimulating children's consumption of legumes? These guiding questions were presented as small stimuli to introduce the subject rather than in the form of questions.

In terms of practical applications, the games were tested by children on two independent occasions: in one school class and at an event on Legume's Day, where children from 50 schools in the Portuguese district of Famalicão were present.

### *Ethical aspects*

The Research Ethics Committee of Universidade Católica Portuguesa approved the research project (N.º 104). All study procedures were conducted in full compliance with the Declaration of Helsinki on ethical principles for research involving human subjects. Upon receiving the invitation to participate in the study, the early childhood teachers completed the informed consent form that presented the objectives and procedures of the study.

### *Data analysis*

Data analysis was carried out quantitatively and qualitatively. Quantitative data were presented in absolute numbers and by frequencies. Fisher's exact tests were applied to observe if there were any associations between the independent variables using SPSS 17.0 software (SPSS, Chicago, IL, USA). Statistical significance was defined by a  $p < 0.05$ .

The theoretical and methodological framework used for qualitative data analysis was content analysis. The Thematic Content Analysis proposed by Bardin (1977) was employed

to analyze, interpret, and process the data gathered from the focus group. The analytical process followed the pre-analysis stages, including initial readings and organizing the corpus, consisting of textual and visual transcriptions of the focus group discussions. To maintain anonymity, each participant was assigned a code (e.g., P1, P2, P3) for reference. Subsequently, the data underwent analysis, with relevant excerpts, sentences, or text fragments identified and grouped based on common themes (nuclei of meaning) to form categories. Following categorization, an interpretative synthesis was developed, linking the categories to the research objectives, questions, and assumptions. The researcher who coordinated the study with the help of an external researcher with experience in content analysis carried out the analysis.

#### 5.4 Results

The survey questionnaire received responses from ten teachers representing nine institutions. Eight of these schools were in the North of Portugal, and one was from the central region. Out of the ten, six (66.7%) were public schools, and three (33.3%) were private institutions. Notably, all participating teachers were female.

**Table 5.1** Provision of the vegetarian meal, frequency of legumes on the menu, and teachers' perceived acceptance of legumes by children in public and private kindergartens

	<b>Public</b> <i>n</i> (%)	<b>Private</b> <i>n</i> (%)	<b>Total</b> <i>n</i> (%)	<i>p</i>
Vegetarian meal:				
Yes	6 (66.7)	1 (11.1)	7 (77.8)	0.083
No	0 (0)	2 (22.2)	2 (22.2)	
Frequency:				
Twice a week or less	2 (22.2)	2 (22.2)	4 (44.4)	0.5
Three times a week or more	4 (44.4)	1 (11.1)	5 (55.6)	
Acceptance:				
Yes	3 (33.3)	2 (22.2)	5 (55.6)	1.0
No	3 (33.3)	1 (11.1)	4 (44.4)	

All six public schools reported offering a vegetarian meal, typically once a week. Among the three private ones, two do not, and one provides this meal. Although not all

institutions offer vegetarian meals, all the schools reported that legume grains are regularly on the menu, most of them (n= 5; 55,6%) three times a week. Just over half (n= 5; 55,6%) of schools reported good acceptance. In the present study's sample, no significant associations were observed between the type of institution (public or private) and the provision of vegetarian meals, the frequency, and the acceptance of legume grains. Concerning food education activities, almost all educators (n= 8; 89%) reported that they carried out these actions, except for one private school (Table 5.1).

The content analysis allowed the identification of three categories of legume consumption and three categories of material evaluation, which were:

- About legume consumption: 1) Vegetarian Meals, 2) Acceptance of legume grains, and 3) Factors (positives and negatives) that determine consumption.
- About material evaluation: 1) Material's quality; 2) Awareness; 3) Effectiveness of the activities.

Discussions among the teachers revealed a sense of uncertainty and divergence regarding the provision of vegetarian meals in kindergartens, often linked to children's reluctance to accept these meals: "I disagree (with offering vegetarian meals) (...) vegetables and everything else, yes, but **vegetarian** (dishes), **no** (...)" (P6 - public school); "I think it's very good. But then if there is no continuation of these dynamics at home, or more often at school (...) **there are kids who do not eat**" (P3 - public school); "I don't disagree. We have experience and kids eat (...) **on the vegetarian day they eat**" (P4 - public school). Despite this divergence, teachers from one of the private schools that do not offer vegetarian meals on the menu expressed interest in making this option available: "Next year, we are already talking about **having a vegetarian day**. I don't know if it will go ahead or not" (P2- private school).

Regarding acceptance, data agrees with the quantitative results, which evidenced talks referring to good acceptance (n= 3; 50%) and low acceptance (n= 2; 33,3%) (Table 5.2). Furthermore, in the discussions on the factors determining children's consumption of legume grains, the main facilitators and barriers were identified, which revealed possible paths to promote the consumption of legumes in kindergartens (Table 5.3). Family involvement in the food education process was one of the main facilitating factors (n= 2; 33,3%) that had a broader agreement among the educators (observed by the non-verbal expressions of the participants). The other facilitator most cited was knowing how to prepare and cook legume grains (n= 2; 33,3%). For the school food environment, the information

was divergent. One of the educators (16,7%) reported that children often did not eat at home but ate at school (Table 5.3). On the other hand, another educator pointed out that only those children who ate at home also ate at school: “*It seems that few used to it (eat legume grains) since they eat at home... Because otherwise, we can't do it*” (P1 - private school).

While good preparation and culinary skills were cited as factors that favor the consumption of legume grains, preparing meals in different places from where they will be served (transported meals) was cited as one of the main barriers to their acceptance (n= 3; 50%). Another negative factor mentioned often by the educators was the lack of habit formation of consuming legume grains (n= 3; 50%), evidencing the need to introduce these foods into children's diets as soon as possible. Lack of knowledge (n= 1; 16,7%), cost (n= 1; 16,7%), and lack of time to prepare (n= 1; 16,7%) also were mentioned as barriers (Table 5.3).

**Table 5.2** Acceptance of legumes in Portuguese kindergartens

Category	Attitude	Frequency n (%)	Nuclei of meaning
Acceptance of legumes			“ <i>What we have been noticing is that <b>they prefer legumes to vegetables</b>...they eat more green beans, black beans. They like black beans. And if it's a sprout or a carrot, they often don't want to eat</i> ” (P2 - private school)
	Positive	3 (50)	“ <i>(...) there was a phase (...) were terrible, and <b>now they eat more beans</b>, (...). Yeah, they didn't. It's the idea I have</i> ” (P6 - public school)
			“ <i>By the way, <b>this year my kids ate everything</b>, all the plant foods. And there were many who asked to repeat.</i> ” (P5 - public school)
	Negative	2 (33.3)	“ <i>We know that many times (...)legumes are (...) oh the peas, oh the grain</i> ” ( <b>expression of rejection</b> ) (P3 – public school)
			“ <i><b>They separate everything</b>. At least that's the experience I have</i> ” (P1 – private school)

**Table 5.3** Factors that affect the consumption of legumes in Portuguese kindergartens.

Category	Attitude	Frequency n (%)	Nuclei of meaning
Factors that determine consumption	Positive: knowing how to prepare and cook legumes	2 (33.3)	<p>“(…) <i>I think the big factor is the <b>quality of the preparation</b>. (…)<i> for example, I can't cook lentils the way she cooks (…)<i> it's really the preparation and the way it is presented...</i>” (P4- public school)</i></i></p> <p>“<i>Yeah, exactly. <b>That must be seen</b>” (P3 - public school)</i></p>
	Positive: Family involvement	2 (33.3)	<p>“<i>I think the material is very good, <b>but it should reach the parents</b> (…)<i> without a doubt, they are the ones who need it” (P6 – public school)</i></i></p> <p>“(…) <i>a few years ago, I did a project in kindergarten (…)<i> concerning the articulation with the family, it was very important, I think <b>it was very important for us to have debates with the parents</b> (…)<i> I think there must be a great articulation, (…)<i> with the parents (emphasis) it was completely different (…)</i>” (P3 – public school)</i></i></i></p>
	Positive: school food environment	1 (16.7)	<p>“<i>Often <b>at school, they eat, but at home, they don't</b>” (P6 - public school)</i></p>
	Negative: transported meals	3 (50)	<p>“(…) <i>our cook also cooks for other schools, she cooks almost a thousand meals a day, and there are only 300 of us, so she cooks outside. <b>I don't know how it is getting to the other schools</b> (…)</i>” (P4 – public school)</p> <p>“<i>With so many meals coming out. <b>It's the rice and beans that come like this (mixing gestures with the hands and expression of rejection)</b> (…)<i> it's not easy. Who cook starts preparing meals from 9 am,</i></i></p>

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		<i>and the distribution of meals is at noon...” (P6 – public school)</i>
		<i>“It comes from outside, isn't it? I think that when it is done in the institution... we already have had this situation, <b>with our cooks they ate much better...</b>” (P1 – private school)</i>
Negative: Lack of knowledge	1 (16,7)	<i>“I think the biggest barrier for me is <b>the lack of knowledge about the food value (...)</b>” (P4 – public school)</i>
		<i>“We were here trying to understand what the big barrier is, why they... and maybe us too... Me, for example, I do not eat lentils at home (...) <b>Maybe I was never have had the habit</b>” (P3- public school)</i>
Negative: Lack of habit formation	3 (50)	<i>“Me neither. <b>I was never have had the habit (...)</b> I think that for children start eating, they must start very early knowing this type of taste” (P6 – public school)</i>
		<i>“I think it is a bit of (...) <b>to introduce these habits</b>” (P1 – private school)</i>
Negative: Cost	1 (16.7)	<i>“Then comes <b>the cost of meals</b>, isn't it? At this cost, it is almost impossible” (P1 – private school)</i>
Negative: Lack of time	1 (16.7)	<i>“It is the fast-paced life that also makes it difficult, people come home and probably <b>make faster meals...</b>” (P1 – private school)</i>

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Regarding the material evaluation, most teachers mentioned clarity and objectivity as the main material qualities. One of the participants pointed out that the material was adequate to the curricular guidelines for preschool. Also, three educators revealed that the information contained in the material made them aware of the topic. Four educators' statements indicated that the activities are executable and effective. As negative points, one participant mentioned that more interactive activities made in digital media were missing,

and another pointed out that the games could not be adequate for younger children (Table 5.4).

In the execution of the games, 12 children aged 6 years old participated at school, and about 350 children aged 6-10 years participated in the Legume’s Day event. It was observed that the children reacted very well to the proposed activities. Some didn't know what legumes were, so they got to know them and soon started to play. They liked the games, showed interest in participating, and resisted finishing them (Figure 5.2).

**Table 5.4.** Material evaluation by early childhood educators.

Category	Attitude	Frequency n (%)	Nuclei of meaning
Material’s quality	positive	5 (83.3)	“(…) I thought it was really <b>feasible, understandable, perceptible</b> ” (P3 – public school)
			“(…) information was so <b>clear, so concise, so evident</b> (…)” (P4 – public school)
			“ <b>Accessible, very accessible</b> ” (P1 – private school)
			“ <b>Also objective</b> ” (P5 – public school)
			“From my point of view, it also <b>meets</b> (…) <b> the curricular guidelines for preschool</b> (…)” (P3 – public school)
Awareness	positive	3 (50)	<p>“It was <b>relatively easy to get this idea, to raise awareness</b> (…)”(P3 – public school)</p> <p>“I even <b>started to consume lupin, which I did not</b>” (P4 – public school)</p> <p>“I found it curious that the production of these legumes use less land, less water, and fewer pesticides than products that we ingest daily. <b>This is an important message to convey to children as well. It is a fact of being good to them and to the environment</b>” (P5 – public school)</p>

<b>Effectiveness of the activities.</b>	positive	4 (66.6)	<p><i>“Start by getting to know them (legumes) because they probably only know a little else apart from beans and peas. They start by knowing; just <b>the fact of knowledge</b> is enough; it <b>already attracts them.</b>”</i> (P6– public school)</p> <p><i>“It becomes a visual habit after it becomes a manual habit and then ... <b>they will taste</b> (...)”</i> (P3 – public school)</p> <p><i>“Also about games, I found it very pertinent, and I <b>thought they were easy</b>... to do with the children, according to ages (...)”</i> (P3 – public school)</p> <p><i>“(...) it was a bit like the activities we did (...) <b>more at the sensory level</b>, realizing that they were handling both raw and cooked food and <b>they began to try some of these legumes.</b>”</i> (P2 – private school)</p>
	negative	2 (33.3)	<p><i>“Our kids are digital...it just <b>includes a little of digital</b> too, to have both”</i> (P4 – school public).</p> <p><i>“<b>It's not for all ages.</b> A memory game is not for the little ones.”</i> (P6– public school)</p>



**Figure 5.2.** Games developed with children. Memory game (a), in which the objective was to match the cards with varieties of legumes, and bingo (b), in which the objective was to complete the card with different legumes.

## 5.5 Discussion

Although the law requiring the inclusion of vegetarian meals in Portuguese public schools does not extend to kindergartens, all public kindergartens offer them once a week. Based on the data, pulses are a regular presence on the menu, and most schools serve them three times a week. However, acceptance remains relatively low, at 55.6%. In the perception of teachers, there is uncertainty regarding the provision of vegetarian meals in kindergartens. As for the factors that facilitate consumption, the most cited were family involvement in food education, knowing how to prepare and cook legume grains, and the school food environment. The main barriers were preparing meals in places other than where they would be served and the lack of habit of consuming legume grains. The results also indicated that the e-book reached the objective of delivering a clear and direct message, and those activities are viable and effective. The students that tested the games were engaged and could perform the proposed activities.

The adoption of vegetarian meals once a week aligns with the global campaign "Meatless Monday" (or "Free Meat Monday"), which has gained traction worldwide (Semba *et al.*, 2024). This initiative presents a potential solution to mitigate the environmental impact

of school meals without compromising the nutrients provided to children (Blondin *et al.*, 2022). Nevertheless, further studies are warranted to investigate this matter, specifically in Portuguese kindergartens.

The findings revealed that legume grains are consistently featured on the menus of the chosen Portuguese kindergartens, although their acceptance is relatively low. The absence of specific data regarding the serving sizes, coupled with the study's limited scope, underscores the necessity for a more extensive investigation involving a larger and more diverse sample of kindergartens to comprehensively evaluate the frequency of legume grain inclusion in these educational environments.

Meal preparation emerges as a critical factor influencing legume grain consumption, according to insights provided by educators. They emphasized that employing effective preparation techniques can enhance acceptance among children. Furthermore, meal preparation outside school was identified as a barrier to acceptance. Previous observations have highlighted that Portuguese children prefer meals prepared within the school's kitchen facilities over those prepared elsewhere (Cardoso *et al.*, 2019). This preference aligns with recurrent findings in the literature, which suggest that limited cooking skills and unfamiliarity with diverse recipes represent significant obstacles to legume grain consumption (Szczebyło *et al.*, 2020; Duarte *et al.*, 2020; Havemeier *et al.*, 2017).

Conversely, research indicates that interventions to enhance cooking skills positively impact legume grain consumption. Therefore, training chefs and cooks responsible for school meals on preparing appetizing legume grain dishes could significantly enhance acceptance (Havemeier *et al.*, 2017; Palmer *et al.*, 2018). An illustrative example is the EU project TRUE, which produced a children's cookbook in multiple languages for widespread distribution to schools (TRUE project, 2019). Additionally, prioritizing food preparation in the school kitchen whenever feasible is advisable. Alternatively, if meals are prepared elsewhere and transported, it's crucial to minimize the time between preparation and distribution while ensuring the visual appeal and temperature of the dishes served.

During the focus group, nearly all participants highlighted family involvement in school food education as a significant factor contributing to legume grain consumption. This finding is consistent with previous studies indicating that parental engagement plays a vital role in the success of dietary interventions to improve children's eating habits (Van Cauwenberghe *et al.*, 2010). Additionally, participants cited the school food environment as another facilitating factor. Research suggests that school meals are pivotal in shaping children's diets (European Commission, 2021). However, it's worth noting that this influence

wasn't observed in the reality described by one educator (P1). Upon closer examination of this educator's school context, it becomes apparent that legume grain acceptance is low, meal preparation occurs outside the school (transported meal), and notably, this was the only school that didn't implement food education activities. These factors collectively may explain the limited influence of the school food environment on legume grain acceptance in this particular case.

One commonly cited barrier to legume grain consumption is the lack of established habits. A study conducted in Poland identified this factor as the primary obstacle to legume grain intake (Szczebyło *et al.*, 2020). To cultivate healthy eating habits, legume grains should be introduced into infant diets before the age of one (Vieira *et al.*, 2021). Additionally, a lack of awareness regarding the nutritional value of legume grains and insufficient time for preparation were identified as barriers in this study, consistent with findings from other research on this topic (Szczebyło *et al.*, 2020; Duarte *et al.*, 2020; Palmer *et al.*, 2018).

However, it's important to acknowledge several limitations in the present study. Firstly, the sampling method relied on the research team's network, resulting in relatively few participants. Another notable limitation was the execution of only one focus group. Ideally, multiple focus group discussions should be conducted with various groups to identify trends and patterns in participants' perceptions regarding the study's focus (Iervolino and Pelicioni, 2001). These limitations challenge the representativeness of the data and constrain the ability to generalize the results.

## **5.6 Conclusion and implications for research and practice**

The study found that legume grains were consistently featured on the menus of the examined kindergartens, albeit with relatively low acceptance among the children. Results indicated that factors such as legume preparation methods, parental involvement in food education, and early establishment of legume grain-eating habits significantly influenced children's acceptance of legume grains. Therefore, implementing culinary workshops to educate and train school cooks on preparing appealing legume grain dishes and sharing recipes could be effective strategies to enhance acceptance of legume grain-containing meals. Additionally, it's important to prioritize food preparation in the school kitchen whenever possible and introduce legume grains into the nursery menu early on.

Concerning the material, the results suggest that the material produced can be used to promote legume consumption in kindergartens.

To further validate this study's findings, future research should expand the investigation to include a larger number of kindergartens, ideally representing the entire country. This broader scope would provide more comprehensive insights into the factors influencing legume grain consumption in kindergarten settings.

**PART IV.**  
**Discussion and final remarks**

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## **Chapter 6. General Discussion, conclusion, and perspectives**

Pulses play an essential role in the sustainability of the European agri-food system. Thus, the present thesis sought to identify areas to promote legumes, and children's diets were considered a priority due to their potential long-term impact. Viable measures were identified, selected, developed, and validated. This chapter discusses the thesis in general, presenting the potential of these measures to promote legumes in the Portuguese agri-food system and finalizing the thesis with conclusions and perspectives for future research.

## 6.1 Discussion

The Portuguese population is demanding nearly three times more resources than the country's biocapacity, due mainly to the high consumption of meat and seafood. Thus, it's fundamental to re-orient dietary choices away from animal proteins, moving toward legumes and other vegetables (Galli *et al.*, 2020). Also, evidence suggests that pulses can be crucial in achieving the European Farm to Fork Strategy goals, especially in reducing fertilizer use (Costa *et al.*, 2021). Recognizing the contribution that legumes and pulses can have to environmental balance, human health, and the resilience of agricultural systems (Ferreira *et al.*, 2021), it is essential to establish a national strategy to promote the production and intake of these crops within a framework of ecological transition.

Although there has been a 40% increase in the area under legume cultivation in Portugal over the last decade (Pereira *et al.*, 2023), the average degree of self-sufficiency of the main legumes has been estimated at only 18% (Hamann *et al.*, 2019). The Strategic Plan for the Common Agricultural Policy (CAP) submitted by Portugal to the European Commission at the end of 2021 includes support for cultivating protein crops (República Portuguesa, 2022). The materialization of this plan will demand a set of policies developed simultaneously for stimulating and supporting local pulse production, utilization, and consumption. Financing, rural extension, the recovery of traditional varieties, and value creation are measures pointed out as a priority for pulses (Balázs *et al.*, 2019, 2021; FAO FSN Forum, 2016).

The central pillar for sustainability under the CAP for Europe is financing through the Green Direct Payment, which involves rewarding farmers for complying with sustainable agricultural practices, such as maintaining permanent grassland and diversifying crops (Caleman *et al.*, 2017). This strategy can encourage producers to adopt the sustainable cultivation of pulses in consortium with other crops. In addition to financial incentives, the increase in pulses cultivation will occur as new outlets with greater added value are established (Smadja *et al.*, 2019). Thus, pulse value creation should occur simultaneously with the cultivation stimulus. Value creation involves producing a commodity for a particular market (Desire *et al.*, 2021). The current work intended to suggest an initial direction to pulses' value creation in Portugal. From the enrichment of children's foods (in this case, a yogurt added with white lupin flour) and actions in kindergartners, it is possible to add value and create a demand for pulses with positive impacts on environmental and social, and economic dimensions. Previous chapters produced evidence that indicates the viability of these measures.

According to chapter 2, lupin was considered the most suitable pulse to enrich a children's yogurt, based on functional potential and taste. In chapter 3, the yogurt's prototype was presented, and in chapters 3 and 4, its characterization was made. The lupin yogurt resulted in a product with an excellent nutritional profile, suitable texture, and regular sensory profile. More research and formula improvement are still necessary, but the results indicate that lupin-enriched yogurt may be a viable way to boost a local pulse-based value chain.

The food industry can be one of the key players in boosting the cultivation of a legume, from innovations that identify new markets and create greater added value (Smadja *et al.*, 2019). A value chain begins with the production of a primary commodity, followed by processing, delivery, wholesale, and retailing, ending with the consumption of the final product (Bellù, 2013). A study identified four distinct ways to value legumes in Europe. It suggests that in legume-based value chains, usually food-oriented, launched by extension services or traders, the stakeholders have more positive perceptions of the legumes business context (Smadja *et al.*, 2019).

Even though white lupin (*Lupinus albus* L.), yellow lupin (*L. luteus* L.) and narrow-leaved lupin (*L. angustifolius* L.) are native cultures in Europe, its cropping area remains modest, with insufficient production to guarantee the stable and sufficient supply (Lucas *et al.*, 2015). In Portugal, annual lupin production has been around 6 tons since 1990 (<https://www.fao.org/faostat/en/#data/QCL>). Insufficient yield in certain areas constitutes a significant barrier to the expansion and economic sustainability of lupin growth. However, scientific, and technological investments may allow lupin cultivation into a broader range of climatic conditions across Europe (Lucas *et al.*, 2015). The cultivation of lupin in Portugal would be stimulated by lupin yogurt production. Here, we did a simulation of the lupin amount that would be demanded from the production of lupin yogurt. For this calculation, we considered the survey "Balança Alimentar Portuguesa" which revealed that, in 2020, all the Portuguese population consumed an average of 21.1 kg/person of yogurt (INE, 2021). In a population of about 10.3 million in 2020 (INE, 2021b), yogurt consumption was nearly 217330 tons. Thus, to estimate the lupin demand from the lupin enriched yogurt production, we established a hypothetical situation where only 1% (2173 tons/year) of all yogurts consumed in Portugal were enriched with lupin flour at a concentration of 4%. It resulted in a demand for 90 tons of lupin per year. Therefore, there is an opportunity to considerably raise national production by the creation of a lupin yogurt value chain. Even though the lupin

enriched yogurt was developed having children in mind, it does not impede from being consumed by the general population.

Chapter 5 observed pulse consumption in selected Portuguese kindergartens and evaluated a material produced to support teachers in food literacy for pulse consumption. This chapter's results showed that pulses are frequent in kindergarten menus, evidencing a permanent demand to be explored by procurements, overall, in public schools. Also, the material produced, together with other actions such as pulse cook skills by training school cooks on preparing tasty legumes and parents' enrolment, have the potential to improve acceptance, promoting pulse consumption by children.

Over the past decade, governments worldwide have been developing public food procurement (PFP) initiatives that use public purchasing power as a policy instrument to promote sustainable development, which is named sustainable public procurement (SPP) (FAO, 2021). The large government purchases give public procurement the power to influence both food consumption and food production patterns, shaping markets and regulating market players to deliver multiple social, economic, and/or environmental benefits (Malacina *et al.*, 2022; FAO, 2021). In high-income countries, public procurement reaches, on average, 12% of gross domestic product (GDP) (Malacina *et al.*, 2022). In the European Union, SPP debate and practices have advanced. In some states members (Sweden, Finland, Denmark, France, and Italy, for instance), a legal framework has given impulse to the public procurement strategies toward sustainable objectives, addressing local traditional food, organic and healthy food in schools and hospitals (Stefani *et al.*, 2017).

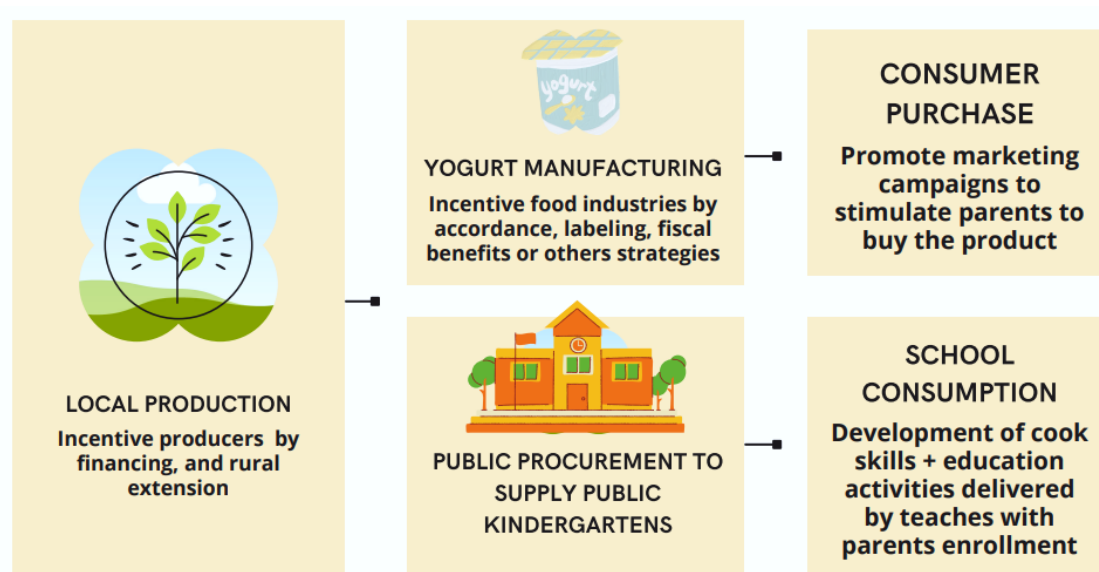
Sustainable food procurement is closely linked to school feeding programs designed to provide children in schools with safe, diverse, and nutritious food sourced locally from smallholder farmers. Purchasing from local smallholders is a strategy implemented mainly in low- and middle-income countries, where the school feeding program is used as a tool to promote the health and well-being of children, as well as to support local agricultural production and promote the economic inclusion of farmers (FAO, 2021).

A program of pulse public procurement to supply kindergartens should support a sustainable productive chain in Portugal, which could promote pulse production and consumption. We simulated the pulse demand that should be created from kindergarten menus. For this calculation, we considered the average amount of beans, which is the pulse most consumed in Portugal (Duarte *et al.*, 2020), recommended by the general direction of education in the kindergarten's menu, which is 10g/ per preschool student/ day (Lima, 2018). As the study pointed the weekly frequency of pulses for most kindergartens is three times a

week, and the daily average offered drops to 6g/ per preschool student. Considering that the number of children enrolled in kindergartens in 2020, in Portugal was 251108 (pordata.pt) and the school year has about 185 days, the total demand is 278.7 tons of beans per year. Knowing that the national production of dry beans fell from around 50000 tons/year in the 1960s to around 2000 tons/year in the last 10 years (<https://www.fao.org/faostat/en/#data/QCL>), the kindergarten demand would stimulate the resumption of dry bean production in Portugal.

From the pulse demand produced by the two measures, value chains should be organized to promote pulses in the Portuguese agri-food system. Of course, in these processes, public sector leadership is essential for implementing coordinated intersectoral actions in articulation with the non-public sectors. The central government would offer governance structures such as human resources, financial and administrative resources, and implement specific incentives and regulations to move on more sustainable agriculture, at both national and local levels. Also, awareness and consumer behavior strategies are essential. Small cities and towns can play a key role in fostering resilient and economically prosperous value chains given their proximity and close interaction with relevant actors enabling the creation of sustainable rural-urban territories (Galli *et al.*, 2020). Figure 6.1 provides an overview of articulating the suggested measures for pulses cultivation and consumption in Portugal. To achieve environmental, social, and economic benefits, the incentive for local production, mainly by funding, technical assistance, training, and physical infrastructure, preferentially aimed at smallholders, should be the first step. After that, stimulations for a value chain creation from integrating smallholder farmers in small and medium agribusinesses to the production of lupin yogurt should be given. Regulatory and non-regulatory measures could encourage the food industry to adhere to this initiative. In addition to this, marketing campaigns targeting parents should be promoted to incentivize parents' purchases.

In parallel, public procurement for kindergartens, of local production, at a fair price should be prioritized. Additionally, a set of actions should be developed to promote pulses acceptance by children. Both measures can create a demand for local production, improving farm sustainability while helping in better nutrition and forming the pulses habit in childhood.



**Figure 6.1** Suggested measures for pulses value creation in Portugal

## 6.2 Conclusion and future research perspective

Implementing a food strategy that seeks to increase the consumption of plant-based proteins by incorporating pulses into Portuguese agricultural systems may help balance diets, promote social inclusion, and improve environmental sustainability. The results in this thesis indicate that the enrichment of children's yogurts with lupin and the promotion of pulses in kindergartens are viable measures that can increase the demand and consumption of pulses.

Enrich children's yogurt with lupin is an alternative to produce a nutritious and perhaps functional product while it may drive a local pulse-based value chain. Additionally, promoting pulses in kindergartens by local procurement and actions that stimulate preschoolers' consumption can favor domestic production with social and economic inclusion and help increment the habit of pulse consumption in the Portuguese population. The increase in pulse demand resulting from these measures can promote environmental gains in the crop system. Also, the probable rise in consumption can improve health and nutrition.

The findings of this work are expected to provide insights for policymakers and inspire the creation and implementation of public policies that boost pulses in the Portuguese agri-food system. Notably, the performance of these policies will require multisectoral arrangements that facilitate coordinated actions, bringing together various actors at the national, regional, and local levels. Otherwise, the results may not be as successful as expected.

Before making food policy decisions, adjustments and additional studies may be required. Alterations in yogurt formulation such as using more refined ground flour, rigorous time and temperature control during processing, and reducing skimmed milk powder can improve sensory aspects and the product's environmental impact. Making these changes and proceeding with new evaluations is recommended. A consequential LCA after formulation improvements could be carried out to observe the environmental impact considering crop rotations. Additionally, studies are required to evaluate mineral bioavailability, antinutritional factors, and stability during the storage of yogurt formula. Also, the possible yogurt functional property should be more investigated. In Chapter 2, it was observed that white lupin flour, at a concentration of 4% and 6%, supports the growth of probiotic bacteria *in vitro*. Additional *in vitro* studies, or ideally human studies, can be developed to see if this capacity of white lupin remains after its incorporation into yogurt and passage through the digestive tract.

Regarding the kindergarten study, amplifying the sample to guarantee the data representativeness and confirm the results is necessary. Also, the practical application in schools of the material developed for the teachers, followed by an evaluation of its effectiveness, is desirable.

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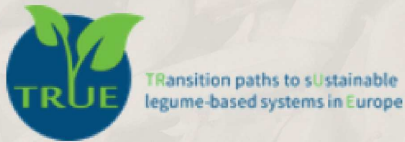
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## **APPENDIX**

E-book: Legume Ação



Escola Superior de  
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Universidade  
Católica Portuguesa.

Porto

A large, stylized graphic featuring the text "LEGUMINE AÇÃO" in a green, hand-drawn font. The word "LEGUMINE" is on the top line and "AÇÃO" is on the bottom line. To the right of the text, there are three colorful illustrations of beans: one yellow, one brown, and one red. The entire graphic is enclosed within a thick, brown circular border.



**Escola Superior de  
Biotecnologia.  
Universidade Católica  
Portuguesa.  
Porto**



Organização dos conteúdos:

**Evla Ferro Vieira**

Organização e projeto gráfico:

**Thayana Araújo**

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# Sistemas alimentares



Os sistemas alimentares compreendem todos os processos envolvidos na origem dos recursos, produção, processamento, transporte, comercialização e consumo de alimentos, incluindo a produção e o descarte de resíduos em todo esse trajeto.

O sistema alimentar que atualmente predomina é ambientalmente insustentável, e não está contribuindo para saúde. Este sistema é responsável por:

 **1/3 das emissões de gases de efeito estufa**

 **70% do consumo global de água limpa**

 **Desperdiçar 1/3 dos alimentos produzidos**

**A comida é o fator mais forte para otimizar a saúde humana e a sustentabilidade ambiental na Terra.**

Uma transformação global na produção e consumo de alimentos é urgente.

Mudanças individuais na dieta serão importantes pois deverão influenciar a demanda por certos alimentos e diminuir a pressão sobre o sistema alimentar global. As dietas baseadas em plantas tendem a apresentar melhores resultados em termos de emissão de gases de efeito estufa, uso de terras, consumo de água e biodiversidade, além de produzirem melhores resultados de saúde. Neste contexto é importante considerar o papel das leguminosas.

# As leguminosas

As leguminosas são plantas da família Leguminosae (ou Fabaceae) que produzem vagens com sementes comestíveis para humanos e animais.

CLASSIFICAÇÃO

## LEGUMINOSAS

### Secas

Feijão, ervilha, fava, grão-de-bico, lentilha, tremoço, feijoca, chícaro

### Oleaginosas

Soja, amendoim

### Frescas

Feijão verde, ervilha



As leguminosas são boas para a saúde e amigas do ambiente

Em rotação com outras culturas, as leguminosas têm uma capacidade de fixar no solo o azoto proveniente da atmosfera, o que reduz a demanda por fertilizantes sintéticos. Essa autossuficiência representa uma vantagem ambiental, uma vez que o uso de fertilizantes sintéticos pode ser responsável por até 65 % da pegada de carbono de cada cultivo. Além disso, o cultivo de leguminosas aumenta o sequestro de carbono pelo solo, o que significa absorção parcial do carbono atmosférico.

**1 kg**  
carne  
bovina



**1 kg**  
proteína  
dos feijões

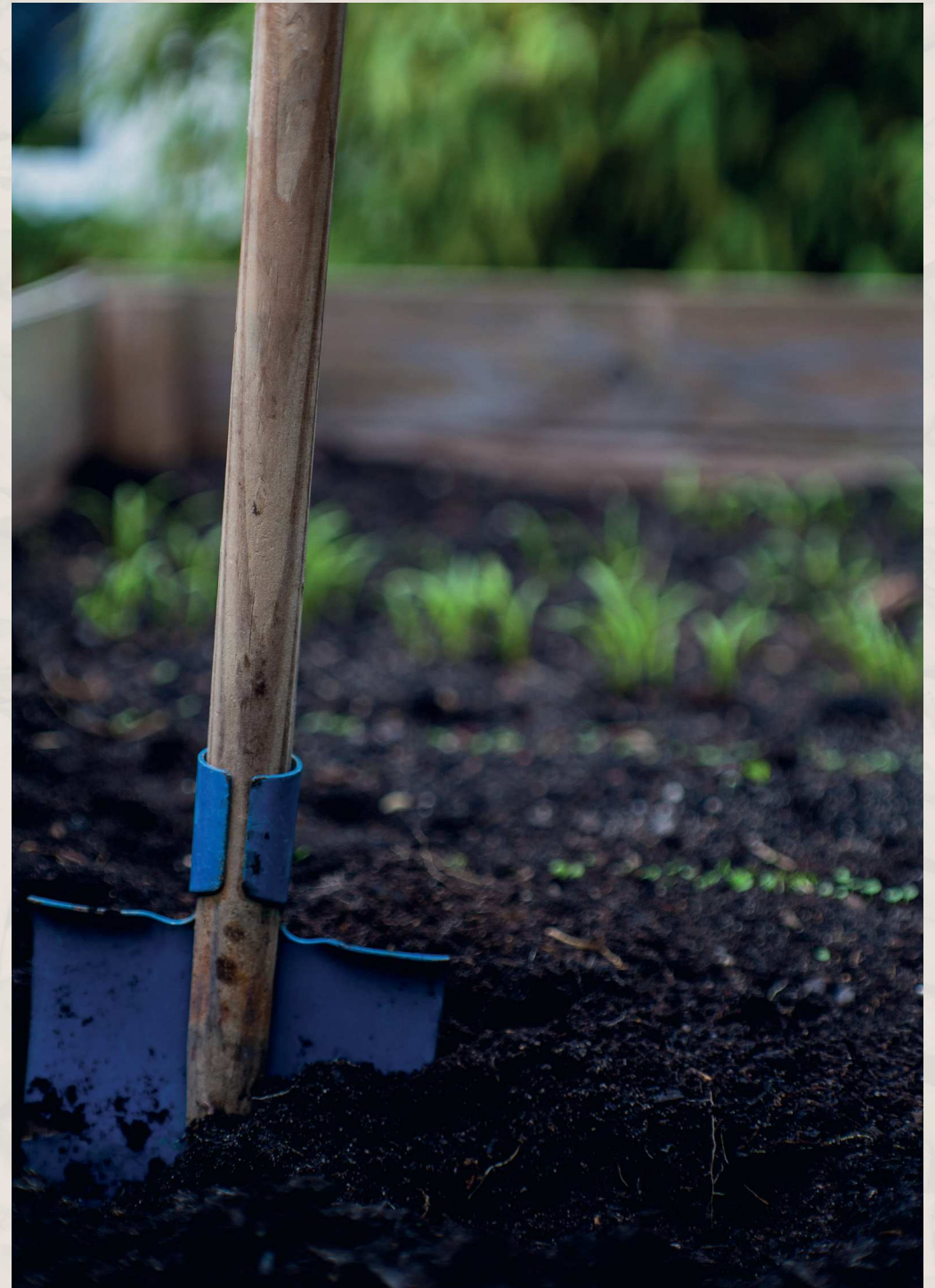
Comparando 1 kg de proteína de carne bovina com 1 kg de proteína dos feijões, as leguminosas precisam de:

- 18x menos terra;
- 10x menos água;
- 12x menos fertilizantes sintéticos;
- 10x menos pesticidas;
- Além de emitirem 250x menos gases de efeito estufa.

As leguminosas também promovem a biodiversidade, recuperam ou melhoram a fertilidade do solo, têm baixa necessidade de água e são altamente resistentes a doenças e pragas.

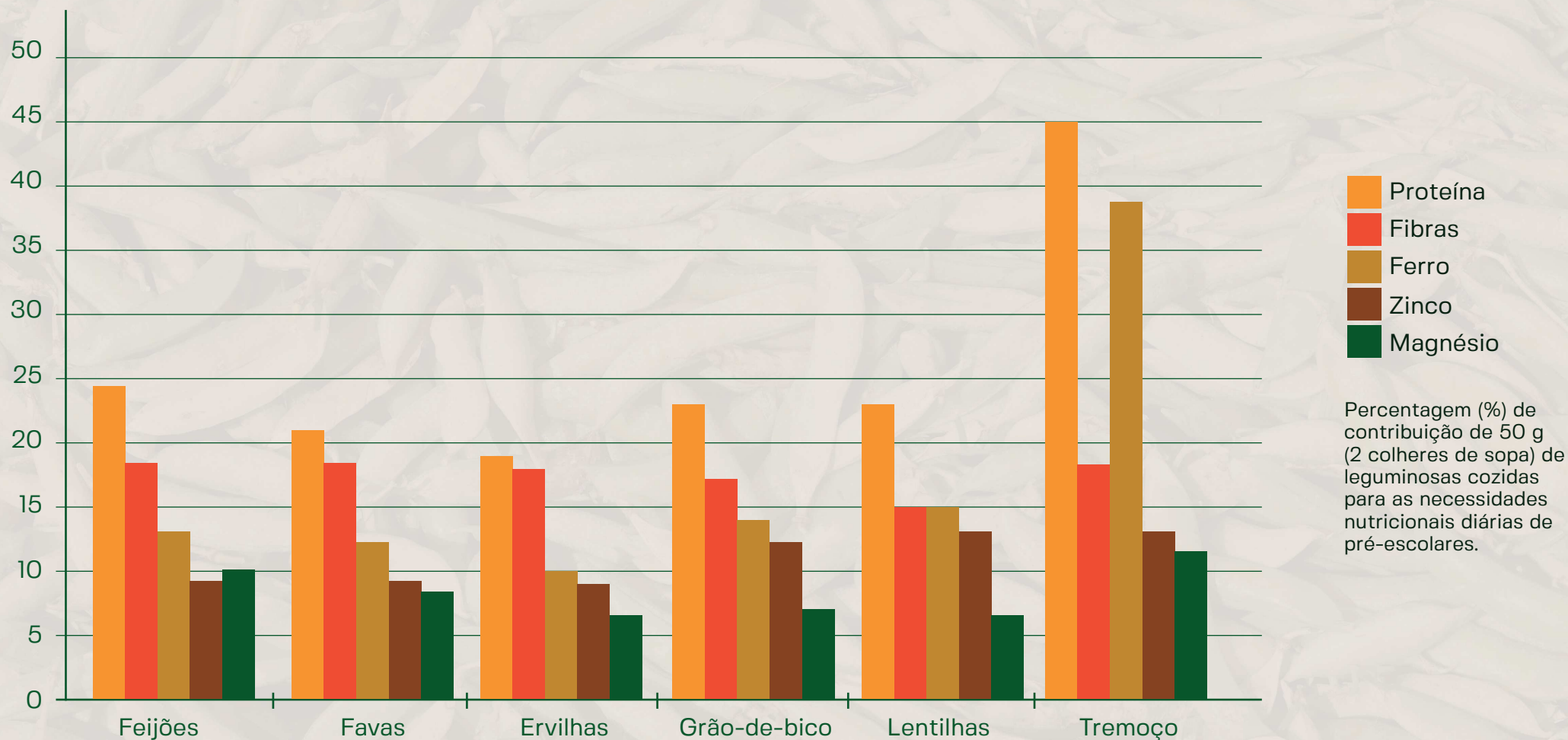
As leguminosas proporcionam elevados benefícios para a saúde, como a prevenção e controlo de doenças crónicas (Ex. obesidade, diabetes, doenças cardiovasculares e cancro).

Adicionalmente as leguminosas são excelentes fontes de proteínas de baixo custo, e mantêm seus benefícios para a saúde por longos períodos de armazenamento. Elas podem substituir parcialmente as proteínas animais em dietas à base de plantas, proporcionando ganhos ambientais e de saúde.



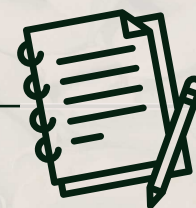
# As leguminosas na alimentação das crianças

As leguminosas contém na sua composição boa quantidade de fibras, vitaminas do complexo B e minerais, como ferro, o zinco, o magnésio, o potássio e o fósforo. Pela composição nutricional, são alimentos muito importantes em períodos de rápido crescimento, como na infância.





As leguminosas podem entrar na alimentação dos bebés a partir dos 8 meses. O consumo de leguminosas nessa fase é fundamental para a aceitação destes alimentos no futuro.



Nota: As leguminosas têm na sua composição substâncias que podem restringir a absorção de nutrientes ou causar desconforto gastrointestinal. Estes fatores podem ser reduzidos mediante estratégias de preparação que diminuem o conteúdo destas substâncias:

- Demolhar antes de confeccionar;
- Trocar a água 1 ou 2 vezes durante a demolha;
- Rejeitar a água da demolha;
- Enxaguar as leguminosas enlatadas.



# Quantidades recomendadas

As leguminosas compõem um grupo com o seu próprio nome. A Roda dos Alimentos recomenda a ingestão de:  
1 porção por dia para as crianças de 1 a 3 anos  
1 a 2 porções por dia a partir dos 4 anos

## Uma Porção de Leguminosas corresponde a:

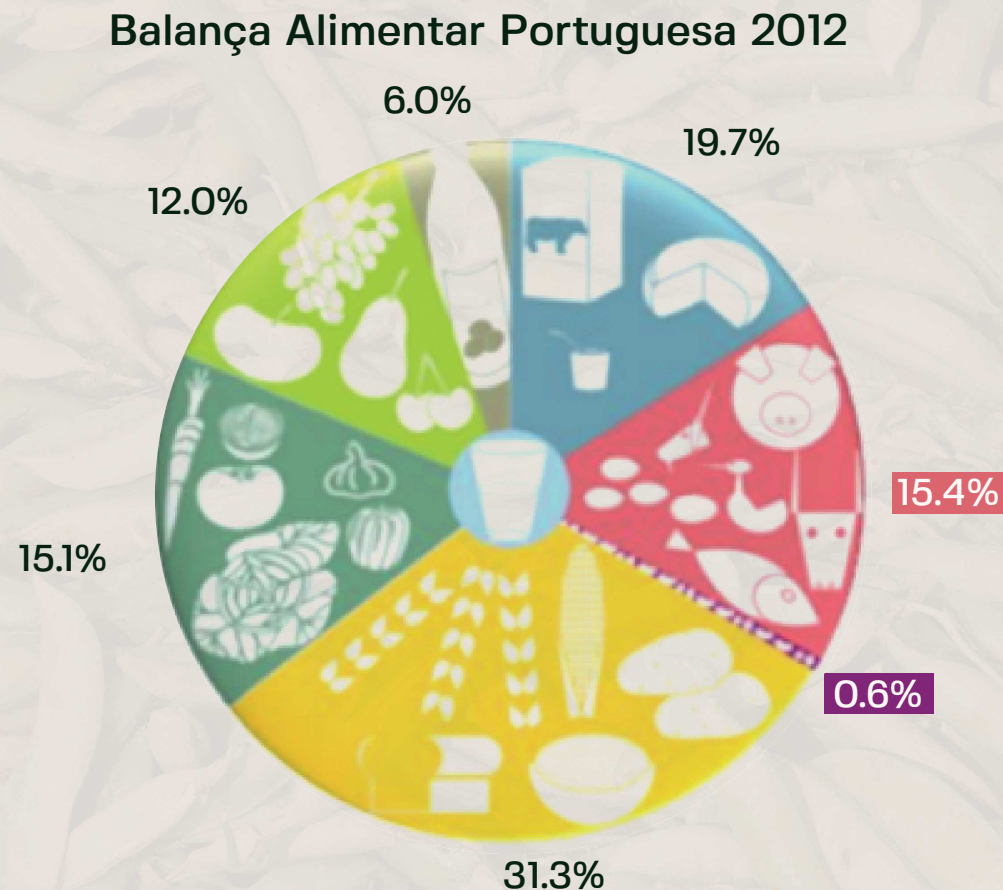
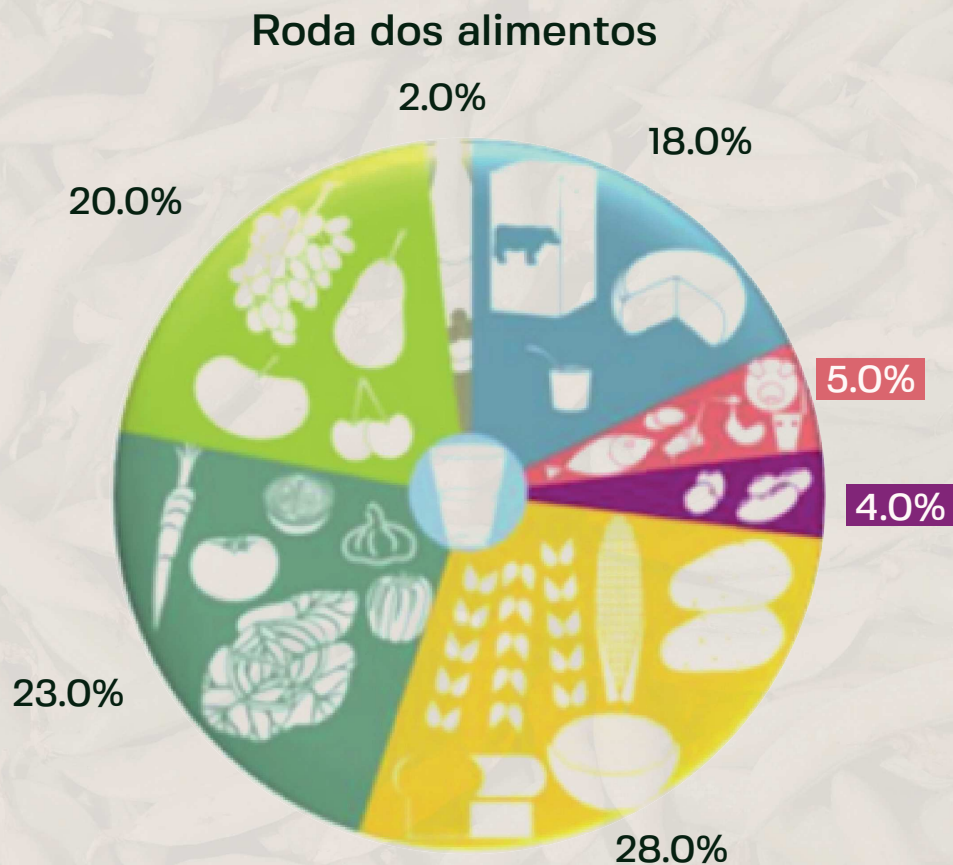
- 🍴 1 colher de sopa de leguminosas secas cruas (ex: grão-de-bico, feijão, lentilhas) (25 g);
- 🍴 3 colheres de sopa de leguminosas frescas cruas (ex: ervilhas, favas) (80 g);
- 🍴 3 colheres de sopa de leguminosas secas / frescas cozinhadas (80 g).



Leguminosas

← **1 a 2 porções por dia**

# Quantidades recomendadas × Quantidades consumidas



A promoção de dietas mais sustentáveis reduzidas em proteína animal e aumentadas em proteína vegetal, especialmente leguminosas, deve começar na infância, que é um período crítico para o desenvolvimento das preferências alimentares.

Figura adaptada da Roda dos Alimentos da Direção Geral do Consumidor  
Fonte: Associação Portuguesa dos Nutricionistas



## A promoção de hábitos alimentares saudáveis na infância

A infância é uma fase crítica para a formação de hábitos alimentares com repercussões ao longo da vida. Portanto a promoção da alimentação saudável e sustentável deve começar desde muito cedo.

**A dieta das crianças sofre influência de um série de fatores. Dentre estes destacam-se:**

1. Os gostos e preferências da própria criança
2. Os pais e cuidadores
3. O ambiente alimentar escolar

### Gostos e preferências alimentares

As preferências alimentares têm grande influência sobre o padrão alimentar das crianças. É durante o processo de socialização que as crianças desenvolvem a percepção de sabores, começam a ter suas preferências alimentares e formam seu comportamento alimentar. A depender dos alimentos disponíveis e acessíveis nesta fase é possível promover ou dificultar o consumo de uma alimentação adequada.

Fatores envolvidos na formação das preferências alimentares:

- Percepção sensorial dos alimentos (predisposição genética + exposição aos sabores desde a gravidez)
- Experiências pós ingestão (se sentiu-se bem ou sentiu-se mal)
- Contexto social (positivos - ex.: festas e comemorações - ou negativos - ex.: ambientes coercitivos; convivência com os pares ou com adultos que são referência)

## Os pais e cuidadores

Os pais e cuidadores são quem inicialmente determinam os alimentos que serão comprados, preparados e, eventualmente, consumidos pelas crianças. À medida que as crianças crescem, elas moldam de forma mais independente suas próprias decisões alimentares. Então os pais e cuidadores passam a responder às dicas, reações e solicitações das crianças.

## O ambiente alimentar escolar

O Ambiente de alimentação escolar inclui a cultura alimentar da escola, tudo o que é servido na ementa escolar e nas cantinas de venda de alimentos dentro e nos arredores das escolas. Este é talvez o fator externo mais importante na formação de padrões alimentares, especialmente quando as crianças passam a maior parte do dia na escola. Os alimentos disponíveis (ou não disponíveis) na alimentação escolar são capazes de moldar os hábitos alimentares a curto e longo prazo. Além disso, o ambiente escolar é um local extremamente favorável para atividades de educação alimentar.



## Características nutricionais e comportamento alimentar pré-escolar (3-6 anos)

### **Crescimento:**

O ritmo de crescimento é menor quando comparado à fase anterior (0-2 anos)

### **Preferências alimentares (biológicas):**

Doces e salgados. Aversão ao ácido e amargo  
Prefere alimentos com alta densidade energética

### **Preferências alimentares (ambiente):**

Sensível às aversões do cuidador

### **Fatores psicossociais:**

Período de maior aversão a alimentos desconhecidos (neofobia)  
Alta resistência para provar alimentos

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**O Aumento da exposição a alimentos saudáveis nesta fase é fundamental para estimular o consumo, especialmente entre os pré-escolares.** Oferecer o alimento repetidas vezes, em diferentes ocasiões, é uma maneira efetiva de promover a aceitação.

As pesquisas indicam que as crianças aumentam a ingestão de alimentos pouco aceitos (ex.: vegetais) após cinco exposições; No entanto, em média, as crianças geralmente requerem entre oito a dez exposições em um intervalo regular (Ex.:uma vez por semana).

Nesta idade, as atividades sensoriais são muito efetivas para promover a aceitação dos alimentos.

## Sugestões de atividades que estimulam o consumo de leguminosas nas crianças da pré-escola

### Atividades que utilizam os sentidos:

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**AUDIÇÃO:** Ouvir e repetir o nome da leguminosa através de histórias ou músicas; usar as diferentes leguminosas para fabricar instrumentos musicais; games do tipo “quem sou eu?” (quando as crianças já estiverem familiarizadas com o aspeto da leguminosa, descreve-se a sua forma e pergunta de qual leguminosa estamos a falar);



**TATO:** Sentir as diferentes texturas das leguminosas enlatadas e cozinhadas na hora; mosaico de leguminosas em grãos;



**VISÃO:** Explorar as diferentes leguminosas e suas cores por associação das imagens ou mesmo da própria leguminosa ao seu respetivo nome (clique no link para acessar o site: [https://www.bforball.com/kids-pulses.php#google\\_vignette](https://www.bforball.com/kids-pulses.php#google_vignette)); expor a crianças a histórias com imagens de leguminosas;



**OLFATO:** Diferença de cheiro entre diferentes leguminosas ou entre leguminosas enlatadas e cozinhadas na hora;



**SABOR:** Promover degustações de leguminosas preparadas de diferentes maneiras (jogo do sim ou não – pedir para a criança degustar e dar a sua opinião sobre a preparação culinária)

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## **Atividades que utilizam a abordagem “do prado ou prato”:**

Estas atividades também são interessantes para as crianças.  
Sugere-se as seguintes etapas:

- 1) Germinação
- 2) Plantio em horta
- 3) Colheita e processamento (retirada da vagem, seleção, etc)
- 4) Atividades culinárias (clique no link e veja sugestões de receitas para crianças aqui: <https://www.true-project.eu/publications-resources/recipes/translations-easy-peasy/>)

## **Atividades multidisciplinares e culturais**

Leguminosas e seu local de origem: apresentar o mapa do mundo com as respectivas origens das leguminosas (para mais informações consultar: <https://www.true-project.eu/publications-resources/recipes/translations-easy-peasy/>)

Explorar pratos típicos com leguminosas em Portugal e no mundo (<https://www.vortexmag.net/pastel-de-feijao/>)

Links interessantes:

<http://www.fao.org/pulses-2016/news/news-detail/en/c/385357/>

<http://www.bean-world.com/en/welcome/>



## **Jogos com leguminosas**

Jogo da memória (Imprime a página na frente e no verso, e recorta na linha tracejada)



**Feijão preto**



**Feijão preto**



**Tremoço**



**Tremoço**



**Ervilhas**



**Ervilhas**



**Grão-de-bico**



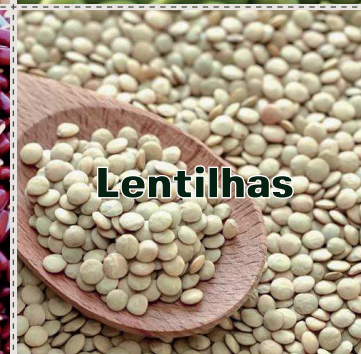
**Grão-de-bico**



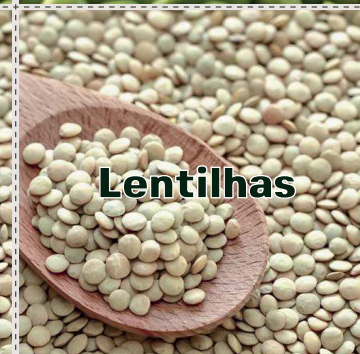
**Feijão azuki**



**Feijão azuki**



**Lentilhas**



**Lentilhas**



**Feijão  
catarino**



**Feijão  
catarino**



**Feijão branco**



**Feijão branco**



**Favas**



**Favas**



**Lentilhas  
amarelas**



**Lentilhas  
amarelas**



**Feijão frade**



**Feijão frade**



**Lentilhas  
vermelhas**



**Lentilhas  
vermelhas**

**JOGO  
DA  
MEMÓRIA**

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### **Jogo do “quem sou eu?”**

Põe as crianças a adivinhar de que leguminosa estamos a falar.

Dica: podes revelar as repostas apresentando as fotografias das leguminosas disponíveis no jogo da memória.



1) **Podes encontrar-me numa deliciosa salada de atum. Tenho uma pinta preta no meu corpo de cor clara. Quem sou eu?**



2) **Os portugueses adoram-me, provavelmente porque sou nutricionalmente muito rico. Tenho uma cor amarela e sou bonito. Quem sou eu?**



3) **Podes comprar-nos congeladas, frescas ou secas. Tenho uma bela cor verde e faço deliciosas sopas. Quem sou eu?**



4) **Estou em vários pratos portugueses, desde as deliciosas feijoadas até o pastel de feijão. Quem sou eu?**



5) **Aparentemente estive demasiado tempo ao sol. Sou a estrela da feijoada brasileira. Quem sou eu?**



6) **Os portugueses gostam de por-me na salada de bacalhau. Mas também posso ser transformado numa deliciosa pasta chamada homus ou num delicioso bolinho chamado falafel. Quem sou eu?**



7) **Na Alemanha chamam-me “feijão gordo” pois sou grande e verde. Nem toda gente gosta de mim pois tenho um sabor “forte”. Quem sou eu?**



8) **Somos uma família de muitas formas e cores diferentes. Somos pequeninas, mas muito versáteis. Podemos ser servidas com enchidos, cenouras e batatas, ou combinadas com especiarias exóticas como o caril.**

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Respostas:

1) Feijão frade; 2) Tremoço; 3) Ervilhas; 4) Feijão branco;  
5) Feijão preto; 6) Grão-de-bico; 7) Favas; 8) Lentilhas

Bingo das leguminosas (Imprime e recorta na linha tracejada)



Bingo das leguminosas (Imprime e recorta na linha tracejada)

<b>Tremoço</b>	<b>Feijão azuki</b>	<b>Feijão branco</b>	<b>Favas</b>
<b>Feijão preto</b>	<b>Ervilhas</b>	<b>Feijão catarino</b>	<b>Lentilhas vermelhas</b>
<b>Grão-de-bico</b>	<b>Lentilhas</b>	<b>Lentilhas amarelas</b>	<b>Feijão frade</b>



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