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Isolation, characterization and use of *Lactiplantibacillus plantarum* LP95 in the production of fermented soybean foods

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Summary

Abstract **1**

Riassunto **3**

Chapter 1

Introduction **5**

1.1 Functional food 5
1.2 Probiotics 7
1.3 Lactic acid bacteria (LAB) 8
1.4 Prebiotics 10
1.5 Postbiotics 10
1.6 Fermented foods 11
1.7 Fermented dairy products 12
1.7.1 Yogurt 13
1.7.2 Kefir 13
1.7.3 Dahi 13
1.7.4 Koumiss 14
1.7.5 Buttermilk 14
1.8 Fermented plant-based products 15
1.8.1 Cereal-based beverages 15
1.8.2 Soybean 16
1.8.3 Fermented soymilk 17
1.8.4 Tofu 18
1.9 Functional proprieties of fermented soy products 19
1.10 *Lactiplantibacillus plantarum* 20

References **23**

Chapter 2

Preliminary screening of *Lactiplantibacillus plantarum* **29**

2.1 Introduction **29**

2.2 Materials and Methods **31**

2.2.1 *Lactiplantibacillus plantarum* cultures 31
2.2.2 Antimicrobial activity 31
2.2.3 Cell protein assay 31
2.2.4 β-glucosidase activity 32
2.2.5 Exopolysaccharide (EPS) production 32
2.2.6 Antioxidant activity (ABTS assay) 33
2.2.7 γ-aminobutyric acid (GABA) production 33
2.2.8 Qualitative screening of biogenic amines production 34
2.2.9 Enzyme profile 35
2.2.10 Statistical analysis 35

2.3 Results **36**

2.3.1 Antimicrobial activity 36
2.3.2 EPS production 37
2.3.3 ABTS Antioxidant activity 37
2.3.4 β-glucosidase activity 37
2.3.5 GABA production 38
2.3.6 Biogenic amine production 39
2.3.7 Enzyme profile of *Lp. plantarum* LP95 39

2.4 Discussion **40**

2.5 Conclusions **43**

References **44**

Chapter 3

Fermented soymilk production by *Lactiplantibacillus plantarum* LP95 ..... **49**

3.1 Introduction **49**

3.2 Materials and Methods **50**

3.2.1 Bacterial culture 50
3.2.2 Chemicals 50
3.2.3 Preparation of soymilk 50
3.2.4 Screening for optimum temperature 51
3.2.5 Production of fermented soymilk 51
3.2.6 Determination of Water Holding Capacity (WHC) 51
3.2.7 Flow behavior and apparent viscosity 51
3.3 Chemical analysis 52
3.3.1 Determination of isoflavones 52
3.3.2 Antioxidant activity (ABTS assay) 52
3.3.3 ThioBarbituric acid Reactive-Substances (TBARS) assay 53
3.4 Statistical analysis 53

3.5 Results **54**

3.5.1 Fermentation parameters 54
3.5.2 Physical-chemical and microbiological characteristics of soymilk during fermentation and storage 55
3.5.3 Flow behavior and apparent viscosity of fermented soymilk 56
3.5.4 Bioconversion of isoflavones during fermentation 59
3.5.5 Antioxidant activity variations during fermentation 59
3.5.6 Variation of MDA during fermentation 60

3.6 Discussion **60**

3.7 Conclusions **62**

References **64**

Chapter 4

Fermented tofu production by *Lactiplantibacillus plantarum* LP95 ..... **67**

4.1 Introduction **67**

4.2 Materials and Methods **67**

4.2.1 Production of tofu 67
4.2.2 Physical parameters 68
4.3 Chemical analysis 69
4.3.1 Sugars metabolism 69
4.3.2 Amino acid (AA) profile 70
4.3.3 Antioxidant activity (DPPH assay) 70
4.3.4 Total phenolics 70
4.4 Statistical analysis 71

4.5 Results **71**

4.5.1 Fermentation kinetics 71
4.5.2 Physical parameters 71
4.5.3 Quantitative changes in sugars during fermentation of soymilk 72
4.5.4 Bioconversion of isoflavones 74
4.5.5 Free amino acid profiles 75
4.5.6 Antioxidant activity 77
4.5.7 Total phenolic content 78

4.6 Discussion **78**

4.7 Conclusions **82**

References **83**

Final remarks **85**
Published research articles related to PhD thesis **86**

Tables index

Table 2.1 Antimicrobial activity at 37 °C by cell-free supernatant (CFS) from the five *Lp. plantarum* strains against different indicator bacteria 36

Table 2.2 EPS-released (EPS-r), EPS-bound (EPS-b) and EPS-bound related to cell proteins (EPS-b**) 37

Table 2.3 Antioxidant and β-glucosidase activities of *Lp. plantarum* strains 37

Table 2.4 GABA production (pmol GABA/μg BSA Eq.) from the five *Lp. plantarum* strains grown in LFM medium with and without L-glutamic acid (GLU) 38

Table 2.5 Enzymes detected with the API ZYM system 40

Table 3.1 Viable cell count (log CFU/mL) of *Lp. plantarum* LP95 during preliminary fermentation trial of soymilk at 20 °C, 28 °C and 37 °C 54

Table 3.2 Changes in pH during preliminary fermentation trial of soymilk with *Lp. plantarum* LP95 at 20 °C, 28 °C and 37 °C 54

Table 3.3 Changes of viable cell count (log CFU/mL) and pH in soymilk during the fermentation (37 °C) and storage stage (4 °C) using *Lp. plantarum* LP95 as starter 55

Table 3.4 Values of consistency coefficient (*K*) and flow behavior index (*n*) obtained by fitting the flow curves with Ostwald de Waele model 57

Table 3.5 Apparent viscosity at 50 s^{-1} of soymilk during fermentation stage at 37 °C using *Lp. plantarum* LP95 as starter 57

Table 3.6 Apparent viscosity at 50 s^{-1} of fermented soymilk during storage stage at 4 °C using *Lp. plantarum* LP95 as starter 58

Table 3.7 Deglycosylation of soymilk isoflavones into aglycones during the fermentation stage of soymilk using *Lp. plantarum* LP95 as starter 59

Table 3.8 Variations of Trolox Equivalent Antioxidant Capacity (TEAC) in soymilk during fermentation stage using *Lp. plantarum* LP95 as starter 59

Table 3.9 Variations of Thiobarbituric Acid Reactive-Substances (TBARS) in soymilk during fermentation stage using *Lp. plantarum* LP95 as starter 60

Table 4.1 Viable cell count of *Lp. plantarum* LP95 during fermentation of soymilk (log CFU/mL) at 37 °C and of tofu storage (log CFU/g) at 4 °C 71

Table 4.2 Changes in pH of soymilk during the fermentation by *Lp. plantarum* LP95 72

Table 4.3 Variations in sugar content of soymilk during the fermentation by *Lp. plantarum* LP95 73

Table 4.4 Aglycone isoflavones (daidzein, glycitein, and genistein) in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter 74

Table 4.5 L-amino acids content in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter 76

Table 4.6 Antioxidant activity and total phenolic content in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter 78

Figures index

Figure 2.1 Antimicrobial activity of the *Lp. plantarum* strains CFS against different indicator bacteria expressed as inhibition rate (%) 36

Figure 2.2 GABA production from *Lp. plantarum* LP95 in LFM medium with (■) and without (■) L-glutamic acid (GLU)..... 38

Figure 2.3 GABA amounts synthesized from *Lp. plantarum* strains grown in LFM medium with L-glutamic acid (100 μM)..... 39

Figure 3.1 Viable cell count (log CFU/mL) of *Lp. plantarum* LP95 (continuous line) and pH values (dashed line) during fermentation of soymilk at 20 °C (red line), 28 °C (green line) and 37 °C (blue line)..... 55

Figure 3.2 Viable cell count (log CFU/mL) of *Lp. plantarum* LP95 (blue line) and pH values (orange line) during soymilk fermentation (37 °C; 24 hours) and fermented soymilk storage (4 °C; 49 days)..... 56

Figure 3.3 Viscosity curves of soymilk during fermentation using *Lp. plantarum* LP95 as starter at 37 °C (A), and fermented soymilk during storage at 4 °C (B) at different times: viscosity (η) as function of the shear rate ($\dot{\gamma}$)..... 58

Figure 3.4 Apparent viscosity at 50 s^{-1} of soymilk during fermentation using *Lp. plantarum* LP95 as starter at 37 °C (A), and fermented soymilk during storage at 4 °C (B) at different times 58

Figure 3.5 Variations in TEAC expressed as mg Trolox Eq./100 g D.W. (A) and TBARS expressed as μg MDA Eq./100 g D.W. (B) in soymilk during fermentation using *Lp. plantarum* LP95 as starter... 60

Figure 4.1 Flow chart of fermented tofu production..... 68

Figure 4.2 Acidification kinetic of soymilk during fermentation phase (24 hours) using *Lp. plantarum* LP95 as starter..... 72

Figure 4.3 Representative chromatograms of sugars detected by HPLC in unfermented soymilk (black line) and fermented soymilk at 6 (blue line), 12 (pink line) and 24 (red line) hours of fermentation using *Lp. plantarum* LP95 as starter..... 73

Figure 4.4 Quantitative changes in sugars during fermentation of soymilk using *Lp. plantarum* LP95 as starter: glucose, fructose galactose (A); sucrose, stachyose and raffinose (B) 74

Figure 4.5 Aglycone isoflavones (A: daidzein; B: glycitein; C: genistein) in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter..... 75

Figure 4.6 Representative chromatograms of amino acids detected via HPLC in unfermented soymilk (A), fermented soymilk (B) after 24 hours using *Lp. plantarum* LP95 as starter, and tofu after 21 days of storage (C), compared to standard mix (D) 76

Figure 4.7 Analysis of total antioxidant activity in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter, by ABTS (A) and DPPH (B) assay..... 77

Figure 4.8 Variations of total phenolic content (TPC) expressed as mg GA Eq./g D.W. in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter..... 78

Abstract

In recent years, alongside conventional screening procedures for the evaluation of probiotics for human use, the pharmaceutical and food industries have stimulated scientific research to implement further selective criteria for the identification of bacteria with particular characteristics to be used as a dietary supplement or as a microbial culture in the production of functional foods.
At the same time, in the functional food sector, there is a growing focus on the production of products made from plant sources as an alternative to animal products. Nowadays, lactose intolerance, the need for low-calorie diets and the incidence of hypercholesterolemia, have increased the preference for vegan diets, thus orienting consumers towards the choice of alternatives to cow's milk.
Historically, soymilk was the first plant-based milk intended to provide nutrients to the population where cow's milk intake was inadequate and in particular it is a food that is becoming increasingly important in the diet of people allergic to milk proteins and lactose intolerant. In addition to being a high-protein food, soy contains several substances with functional and nutritional properties: fatty acids, vitamins, peptides, minerals, flavonoids, phenolic acids and saponins.
The fermentation of soymilk has been used for thousands of years as a valid strategy to increase the shelf life of the raw material, but today it is also exploited as a biotechnological strategy aimed at improving its organoleptic and nutritional characteristics.
The main objective of the research activities carried out during the three-year PhD was to obtain fermented soy-based products that, in addition to preserving the nutritional properties of soy, had a greater functional value than the raw material.
In the initial screening phase, five strains *Lactiplantibacillus plantarum* (*Lp. plantarum*) strains, isolated from bee bread were examined.
In particular, the following parameters were evaluated:

— antioxidant activity
— antimicrobial activity
— β-glucosidase activity
— γ-aminobutyric acid (GABA) production
— Extracellular Polymeric Substances (EPS) production

Wide heterogeneity was found in relation to the examined properties, confirming that these are strain-specific. Therefore, results suggest that a careful selection within a given species is of utmost

importance to identify appropriate strains for specific biotechnological applications. In particular, it was observed that *Lp. plantarum* LP95 strain was the best performing strain.

Given these premises, in the following experimental phases, *Lp. plantarum* LP95 was used as a starter for the production of a fermented soymilk and for the production of fermented tofu.

Soymilk was fermented with *Lp. plantarum* LP95, at 37 °C, without the use of stabilizers as well thickeners or acidity regulators.

The results obtained showed that fermentation by *Lp. plantarum* LP95 improves some functional properties of soymilk. In particular, the increase in the content of isoflavones in aglycone form after fermentation was the result of β-glucosidase activity against isoflavone glycosides.

The fermentation kinetics of *Lp. plantarum* LP95 in the soymilk substrate showed that, after 24 hours, this strain is able to ferment the monosaccharides and disaccharides naturally present in soymilk, while leaving unchanged the content of galactooligosaccharides (GOS), which act as a prebiotic substrate for the colonization of bacterial species beneficial for gut health. In tofu, after 21 days of maturation, *Lp. plantarum* LP95 maintains good viability (about 10^6 CFU/g) and the final product retains a high nutritional and functional value, due to its content of isoflavones, amino acids, and GABA.

Based on this evidence, *Lp. plantarum* LP95 appears to be a promising candidate as a starter culture to obtain fermented soymilk products with high nutritional and functional value.

Riassunto

Negli ultimi anni, accanto alle tradizionali procedure di screening per la valutazione dei probiotici per uso umano, le industrie farmaceutiche e alimentari hanno stimolato la ricerca scientifica per implementare ulteriori criteri selettivi per l'identificazione di batteri con caratteristiche particolari da utilizzare come integratori alimentari o come coltura microbica nella produzione di alimenti funzionali.

Allo stesso tempo, nel settore degli alimenti funzionali, c'è una sempre maggiore attenzione alla produzione di prodotti ottenuti da fonti vegetali in alternativa ai prodotti di origine animale. Al giorno d'oggi l'intolleranza al lattosio, l'esigenza di diete ipocaloriche e l'incidenza dell'ipercolesterolemia, hanno aumentato la preferenza per le diete vegane, orientando così i consumatori verso la scelta di alternative al latte vaccino.

Storicamente, il latte di soia è stato il primo latte vegetale destinato a fornire nutrienti alla popolazione in cui l'assunzione di latte vaccino era inadeguata, ed è un alimento che sta diventando sempre più importante nella dieta delle persone allergiche alle proteine del latte e intolleranti al lattosio. Oltre ad essere un alimento ad alto contenuto proteico, la soia contiene diverse sostanze con proprietà funzionali e nutrizionali: acidi grassi, vitamine, peptidi, minerali, flavonoidi, acidi fenolici e saponine.

La fermentazione del latte di soia è stata utilizzata da millenni come valida strategia per aumentare la conservabilità della materia prima ma oggi viene sfruttata anche come strategia biotecnologica finalizzata a migliorarne le caratteristiche organolettiche e nutrizionali.

L'obiettivo principale delle attività di ricerca svolte durante il triennio del dottorato è stato quello di ottenere prodotti fermentati a base di soia che, oltre a conservare le proprietà nutrizionali della soia, avessero un maggiore valore funzionale rispetto alla materia prima.

Nella fase iniziale di screening sono stati esaminati cinque ceppi di *Lactiplantibacillus plantarum* (*Lp. plantarum*), isolati da pane d'api.

In particolare, sono stati valutati i seguenti parametri:

— attività antiossidante
— attività antimicrobica
— attività β-glucosidasica
— produzione di acido γ−amminobutirrico (GABA)
— produzione di sostanze polimeriche extracellulari (EPS)

È stata riscontrata un'ampia eterogeneità in relazione alle proprietà esaminate, confermando che queste sono specifiche del ceppo. Pertanto, i risultati ottenuti suggeriscono che un'attenta selezione all'interno di una determinata specie, è di estrema importanza per identificare ceppi appropriati per specifiche applicazioni biotecnologiche. In particolare, è stato osservato che il ceppo *Lp. plantarum* LP95 era il ceppo con le migliori prestazioni. Date queste premesse, nelle fasi sperimentali successive è stato utilizzato *Lp. plantarum* LP95 come starter per la produzione di un latte di soia fermentato e per la produzione di tofu fermentato. Il latte di soia è stato fermentato con *Lp. plantarum* LP95, a 37 °C, senza l'uso di stabilizzanti, addensanti o regolatori di acidità.

I risultati ottenuti hanno dimostrato che la fermentazione con *Lp. plantarum* LP95 migliora alcune proprietà funzionali del latte di soia. In particolare, l'aumento del contenuto di isoflavoni in forma agliconica dopo la fermentazione è il risultato dell'attività della β-glucosidasi nei confronti dei glicosidi isoflavonici.

La cinetica di fermentazione di *Lp. plantarum* LP95 nel substrato del latte di soia ha dimostrato che, dopo 24 ore, questo ceppo è in grado di fermentare rapidamente i monosaccaridi e i disaccaridi naturalmente presenti nel latte di soia, mentre lasciano inalterato il contenuto di galattoligosaccaridi (GOS), che fungono da substrato prebiotico per la colonizzazione di specie batteriche benefiche per la salute dell'intestino. Dopo 21 giorni di maturazione, il tofu conserva un elevato valore nutrizionale e funzionale per il suo contenuto di isoflavoni, amminoacidi e GABA. Inoltre dopo le prime tre settimane, la vitalità dello starter utilizzato nel tofu rimane elevata (circa 10^6 UFC/g).

Sulla base di queste evidenze, *Lp. plantarum* LP95 può essere considerato una valida coltura starter per ottenere prodotti a base di latte di soia fermentato ad elevato valore nutrizionale e funzionale.

Chapter 1

Introduction

1.1 Functional food

Consumer expectations have evolved significantly in recent decades, particularly with regard to food production. Indeed, an increasing number of consumers believe that food directly affects their health (Mollet and Rowland 2002). Today, food must not only satiate hunger and provide essential nutrients for human health; it must also prevent diseases related to poor nutrition and improve the physical and emotional health of consumers (Siró et al. 2008; Tur and Bibiloni 2016). In these regards, a great importance is assumed by functional foods. Understanding the connection between nutrition and health-particularly at the molecular level-led to the development of the idea of "functional foods", also known as "nutraceuticals" or "pharmaceutical foods", as a novel and useful strategy for achieving optimal health and potentially lowering the risk of disease (Tur and Bibiloni 2016). The concept of functional foods is often mistaken with the notions of nutraceuticals and pharmafoods. Nutraceuticals are substances that are a food-or part of a food-providing medical and/or health benefits, including both prevention and treatment of diseases (Roberfroid 2000; Clemens and Kadharmestan 2018), while the term pharmafoods refers to products on the food-medicine borderline, which first emerged in Japan; they are also called "border foods" as they claim drug-like preventive and therapeutic properties (Tur and Bibiloni 2016). In Japan, the 1980s took place the introduction of the term "functional food" to describe food products enhanced with unique ingredients that had positive physiological effects (Siró et al. 2008). In developed societies, consumers' preferences for certain food products have changed significantly due to growing awareness of the significant relationship between diet and human health. This has led consumers to select certain food products over others in an effort to achieve desired health outcomes (Pappalardo and Lusk 2016). According to Díaz et al. (2020), functional items are great dietary choices since they work to prevent diseases linked to poor nutrition and hence enhance quality of life. Three more requirements in particular have been suggested as extra factors to identify a functional food. In particular, it is a food made of natural elements (rather than a pill, capsule, or powder); it may and should be included in a daily diet; and, when taken, it serves a specific purpose by regulating bodily processes (Rincón-León 2003). Often called functional ingredients, bioactive compounds are biomolecules that are present in both edible and inedible portions of food and agricultural matrices, and have specific physiological benefits. To the group of bioactive compounds belong non-digestible carbohydrates, proteins, peptides, essential amino acids,

parabiotics, postbiotics, omega-3 fatty acids, structured lipids, secondary metabolites and many more molecules from a broad range of living sources (Mondal et al. 2021; Nath et al. 2023). Regretfully, the majority of nations lack a legally recognized definition for functional food; as a result, phrases like medical foods, nutraceuticals and dietary supplements are frequently employed. Thus, natural food enhanced with a health-promoting ingredient, food that has had a component added for specific health reasons, or food that has had an ingredient chemically or technologically modified can all be considered functional food (Adadi et al. 2019; Dobrzyńska et al. 2023).

In fact, since The Food and Drug Administration (FDA) lacks the authority to create a formal regulatory category for functional foods since the Federal Food, Drug, and Cosmetic Act (FFDCA), as amended, does not provide a statutory definition of functional foods. Manufacturers may be subject to one of several current regulatory options when marketing such products. The FDA believes that the current regulatory categories offer flexibility and opportunities for the regulation of so-called functional foods. Manufacturers must take into account the different labelling regulations, safety standards and petition or notification procedures for ingredients in their products when deciding which category to place them in (Ross 2000). According to the European Food Safety Authority (EFSA), a functional food is defined as a food, which beneficially affects one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being, and/or reduction of risk of disease. A functional food can be a natural food or a food to which a component has been added or removed by technological or biotechnological means and it must demonstrate its effects in amounts that can normally be expected to be consumed in the diet (Duttaroy 2019; Dobrzyńska et al. 2023). However, the EFSA does not make any distinction between food supplements and nutraceuticals for beneficial health claim applications for new products and the availability of clinical data to support its efficacy is a necessary condition for any claim authorization (Santini et al. 2018).

The Food and Agriculture Organization (FAO) of the United Nations uses a definition along these lines functional food are “Foods that may provide health benefits beyond basic nutrition” (Spence 2006). A rational definition was proposed by Temple (2022) and affirms as follows: “Functional foods are novel foods that have been formulated so that they contain substances or live microorganisms that have a possible health-enhancing or disease-preventing value, and at a concentration that is both safe and sufficiently high to achieve the intended benefit. The added ingredients may include nutrients, dietary fiber, phytochemicals, other substances, or probiotics”. Even for nutritionists and food experts, defining the boundary between conventional and functional meals can be difficult because most nations lack a legal definition for the expression. Functional food is defined as adding functionality to an already-existing traditional food product

(typically a mainstream product); it does not constitute a distinct category of food in Europe or the USA. However, European legislation considers functional foods as a concept rather than as separate food categories (Coppens et al. 2006; Siró et al. 2008). According to the EU Nutrition and Health Claims Regulation (NHCR) on foods (EC No. 1924/2006), a list of authorised claims has to be published for all member states and nutrient profiles also has to be (Siró et al. 2008), and its was ultimately amended by European Regulation (EU) No. 1047/2012 (Santini et al. 2018). Health claims can be "function claims" and "reduction of disease risk claims" (Siró et al. 2008). The previous mentioned regulation complemented the general principles given in the food labeling Directive 2000/13/EC of the European Parliament and of the Council, and lays down specific provisions concerning the use of nutrition and health claims (Gulati et al. 2014, 2019).

1.2 Probiotics

Over time, the definition of the term "probiotic" has evolved and broadened. The term "probiotic" was coined by Metchnikoff in 1908 and it derives from the Greek/Latin word "*pro*" and the Greek word "*bios*", meaning for life (Iannitti and Palmieri 2010). Metchnikoff created the first definition of a probiotic in 1908 and linked the consumption of fermented milk products to the so-called "longevity effect" (Zendeboodi et al. 2020). Later, in 1956, Lilly and Stillwell (1965) reported that some bacteria are able to produce substances that can promote the development of another microbe. Parker (1974) expanded the definition of "probiotic" in 1974 to encompass substances and organisms that influence the microbial balance in the gastrointestinal system. Fuller (1992) then implemented the concept of probiotic by defining the term probiotic as a live microorganism that positively affects the health of animals by restoring the intestinal microflora. In detail it redefined probiotics as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Since then, the definition of probiotics has been broadened to emphasize the importance of live cells as an essential component of an effective probiotic. According to Havenaar and Huis In't Veld (1992) definition, a probiotic is "a mono or mixed culture of live microorganisms that, when applied to humans or animals, has beneficial effects on the host by improving the properties of the indigenous microflora". The current definition was provided by the Food and Agriculture Organization (FAO) of the United Nations World Health Organization (WHO), according to which probiotics are redefined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO Expert Consultation 2006). Finally, in 2014, the International Scientific Association for Probiotics and Prebiotics (ISAPP) slightly changed the previous definition to "live microorganisms, that when administered in adequate amounts, confer a health benefit on the host"

(Hill et al. 2014). Many bacteria are thought to be potential probiotics. These bacteria come from the genera *Lactococcus, Enterococcus, Streptococcus, Propionibacterium* and *Bacillus*. In particular, several lactic acid bacteria (LAB) are the most important groups of probiotic and food-related bacteria widely used in the food, dairy, and beverage industries. The commonly used strains are from the diverse group of *Lactobacillus* and *Bifidobacterium* which can have a significant influence on health (Latif et al. 2023, 2024). The European Food Safety Authority (EFSA) has provided a list of species presumed to be safe for human consumption in foods under the "Qualified Presumption of Safety" (QPS) concept (EFSA 2007), while the U.S. Food and Drug Administration adopted the term "generally recognized as safe" (GRAS) (Mattia and Merker 2008). A substance may be GRAS only if its general recognition of safety is based on the views of experts qualified to evaluate the safety of the substance (Surve et al. 2022). In regard to their mechanisms of action, Skowron et al. (2022) suggest that probiotic bacteria interact and stimulate the mucosa-associated lymphoid tissue (MALT) immune system, formed, among others, by gut-associated lymphoid tissue (GALT) immune elements. Due to the production of chemokines, cytokines, growth factors, or immunoglobulins, MALT acts as a microbial fighter.

1.3 Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are gram-positive catalase-negative bacteria morphologically appearing as cocci or rods, non-motile, non-sporulating, acid-tolerant, non-respiring but aerotolerant. These bacteria produce large quantities of lactic acid (LA) as a fermentation product of carbohydrate metabolism (Salvetti et al. 2018), that is a water-soluble and highly hygroscopic aliphatic acid (Tang et al. 2023). Lactic acid bacteria generate energy through phosphorylation at the level of substrates, in which the hexoses can then be targeted for homo or heterofermentative metabolism (Mora-Villalobos et al. 2020). In homofermentative LAB's metabolism from one molecule of glucose are produced two molecules of LA and 2 molecules of ATP via the aldolase enzyme, while in heterofermentative metabolism LAB's use the alternative pentose phosphate pathway where hexoses are converted in pentoses and finally produce one molecule of LA and one molecule of ethanol or acetic acid via the phosphoketolase pathway. Additionally, during fermentation LAB produce and release several metabolites, among them organic acids, amino acids, neuroactive molecules, exopolysaccharides, vitamins, and bacteriocins (Tang et al. 2023). Despite LABs are usually auxotrophic for some vitamins, some strains have been demonstrated to be capable of synthesizing B group vitamins, including cobalamin, riboflavin, folates, and thiamine (LeBlanc et al. 2020). During growth phase, some lactic acid bacteria can regulate their cellular density via density-dependent gene expression regulation system, called quorum sensing

(QS). Moreover, QS can regulate many biological functions, such as virulence, bioluminescence, horizontal transfer of DNA, synthesis of antibiotics and bacteriocins, ability to resist to the harsh gastric stress, production of secondary metabolites, formation of biofilms, and alteration of foodstuffs (Li et al. 2021).

LABs use two QS activation pathways that differ in the signal perception mechanism, but use as molecule signal the same peptide, called autoinducer peptide (AIP). The first signaling pathway involves transduction of AIP via a two-component system (TCS). In detail, once the AIP precursors are cleaved and, in some cases, undergone to post-translational modifications, it is secreted into the extracellular environment and binds to transmembrane histidine kinase (HK) which triggers to autophosphorylation; finally, the phosphoryl group are transferred to the specific cytoplasmic transcriptional regulator (RR). The second activation pathway involves the intracellular re-importation of the AIP through oligopeptide Ami (Opp) permease transport system, belonging to the family of ABC transporters. Subsequently, once it has been imported intracellular AIP can interact directly with a cytoplasmic transcriptional regulator belonging to the RNPP family (Rap, NprR, PlcR, and PrgX) and modulate the expression of specific genes (Kareb and Aïder 2020). QS is a pivotal mechanism of bacteria growth since it is responsible of the induction of microbial biofilm formation. Biofilm determines microbial cell adhesion to solid surfaces, which occurs by secretion of high concentrations and a wide range of homo- and hetero-exopolysaccharides (EPSs) (Corsetti and Valmorri 2011; Preda and Săndulescu 2019). Biofilm-producing LAB are promising candidates for the production of pathogens-free food, by competing with pathogenic biofilm-producing bacterial strains. In fact, as it has been reported by Mgomi et al. (2023), they prevent undesirable microorganisms, such as pathogenic biofilms, whose prevention and eradication are severe challenges in a food and clinical environment. Additionally, scientific evidence suggest that EPS have health-promoting properties (Werning et al. 2022) as well as they exhibit a broad range of activities including antioxidants and anti-biofilm agent against pathogenic strains in food and pharmaceutical industries (Xu et al. 2020; Salimi and Farrokh 2023; Yang et al. 2023). Furthermore, EPS may mediate antimicrobial activity by altering bacterial cell surfaces by preventing initial surface binding or by functioning as signaling molecules that control the expression of genes involved in the formation of biofilms (Kim et al. 2010; Silva et al. 2019). Moreover, Rizzello et al. (2014) has also hypothesized a regulatory mechanism of bacteriocin synthesis via QS. With regard to amino acid metabolism, LAB have four pathways, including the arginine deiminase (ADI) pathway, the glutamate decarboxylase (GAD) system, the branched chain amino acid (BCAA) metabolism (Tang et al. 2023).

1.4 Prebiotics

Prebiotics are indigestible food components that specifically promote the growth and multiplication of probiotics, or beneficial, microorganisms already present in the gastrointestinal tract, improving the host's health, physiological, digestive and nutritional needs (Panesar and Bali 2016). In other definition prebiotic activity is “the ability of a given substrate to support the growth of an organism relative to other organisms and relative to growth on a non-prebiotic substrate, such as glucose” (Silva et al. 2019).

A food item must possess three key physiological characteristics to be considered a prebiotic: i) it must be resistant to stomach acid digestion, ii) withstand the breakdown of pancreatic and intestinal enzymes; iii) be fermented specifically by beneficial bacteria in the gut (such as lactobacilli and bifidobacteria) to encourage the growth and/or metabolic activity of these bacteria (Hegazi and Seth 2013). In this sense, adding prebiotics may help encourage probiotic proliferation and improving the probiotic's capacity to withstand gastrointestinal conditions. Currently, the most widely used prebiotics in the food industry are fructooligosaccharides (FOS), galactooligosaccharides (GOS), and trans-galactooligosaccharides (TOS) (Feng et al. 2023). Finally, among their biological activities, the above mentioned EPS also acts as prebiotics, and in fact are highly valued components in the food industry (de Palencia et al. 2009).

1.5 Postbiotics

The International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2019 reviewed the definition and scope of postbiotics and defined it as i) “soluble factors secreted by live bacteria, or released after bacterial lysis, such as enzymes, peptides, teichoic acids, peptidoglycan-derived muropeptides, polysaccharides, cell-surface proteins, and organic acids”; ii) “non-viable metabolites produced by microorganisms that exert biological effects on the hosts” and iii) “compounds produced by microorganisms, released from food components or microbial constituents, including non-viable cells that, when administered in adequate amounts, promote health and wellbeing” (Gurunathan et al. 2023). According to Rafique et al. (2023), postbiotics are substances and/or components of cell walls that are either created by living bacteria or released following bacterial lysis, and they have a positive effect on the host. Through the transfer of certain physiological benefits, postbiotics may benefit host health even though the exact pathways are still unknown. The mechanisms underlying the ability of a postbiotic to mediate an effect on the health of the target host may be multiple, as it may consist of a heterogeneous mixture of components. In particular, Salminen et al. (2021) reported the following five mechanisms of action of postbiotics: i) modulation of the resident microbiota; ii) enhancement of epithelial barrier functions; iii)

modulation of local and systemic immune responses; iv) modulation of systemic metabolic responses; and v) systemic signalling via the nervous system. Furthermore, any claims regarding health benefits would have to be approved by the FDA. These claims could be classified as non-approved general function, which describe a food's influence on the normal structure or function of the human body, or as health claims, which describe a food's ability to lower the risk of disease.

1.6 Fermented foods

According to biochemists, fermentation is “an ATP-generating process in which organic compounds act as both donors and acceptors of electrons”. This definition may be useful for the anaerobic lactic and ethanol fermentations, but is not applicable to many other food fermentations (Marco et al. 2021). Fermented foods are defined as “foods or beverages produced through controlled microbial growth and the conversion of food components through enzymatic action”. In the past, fermentation of food was used as a preservation technique because it reduces the possibility of contamination by pathogenic microorganisms by producing antimicrobial metabolites such as ethanol, bacteriocins, and organic acids. Several foods, are inedible without fermentation, so fermentation is also used to improve organoleptic qualities (taste and texture). Historically, a wide variety of foods including dairy, vegetables, fruits, cereals, legumes like soybeans, meat, and fish have been fermented. Numerous factors influence the fermentation process, such as the microorganisms, nutritional components and ambient circumstances, resulting in thousands of distinct fermented food varieties (Dimidi et al. 2019). In addition to these qualities, it is increasingly recognized that some fermented foods improve human health in ways that are not directly related to the original ingredients. In other words, the products of fermentation and in particular the role played by bacteria, may offer benefits beyond mere sustenance (Marco et al. 2017). The definition includes foods and beverages that are produced by fermentation but might not have living microorganisms at the time of consumption (Marco et al. 2021).

Interestingly, Capozzi et al. (2017) pointed out that numerous researches clarify the nutritional and microbiological importance of fermented foods and beverages in various continents and proposes to broadly classify fermented foods and beverages into nine main classes, based on the type of matrices (raw materials) undergoing fermentation. Fermented foods include cereals, vegetables and bamboo shoots, pulses, roots and tubers, fermented dairy products, fermented and preserved meat products, fermented, dried and smoked fish products, various fermented products and alcoholic beverages. Throughout history, various fermented food and drink products have been developed around the world, such as cheese, bread, yoghurt, soy sauce, vinegar, wine, curd, butter, yukult, fermented sausage, as well as a wide variety of traditional foods (Durazzo et al. 2022).

Microorganisms involved in fermentation are influenced by the composition of raw materials and process parameters (Mudoor Sooresh et al. 2023). Firstly, food can ferment naturally; this process is also known as “wild ferment” or “spontaneous ferment”, in which case the bacteria are naturally present in the raw food or in the processing environment. Secondly, a process known as “culture-dependent fermentation” allows food to be fermented by adding starter cultures (Dimidi et al. 2019). During the fermentation of food, several changes occur, including the breakdown by microorganisms of fermentable carbohydrates into end products such as organic acid, carbon dioxide, and alcohol, as well as antimicrobial metabolites such as bacteriocins that increase food safety by killing or inhibiting food-borne pathogens. Moreover, fermentation improves the organoleptic qualities of food, the digestibility of proteins and carbohydrates, and the bioavailability of vitamins and minerals. It also lengthens the shelf life of food and reduce toxins and anti-nutrients (Farnworth 2005; Şanlier et al. 2019).

Noteworthy, the fermented foods provide new compounds that may also have positive effects on health. In detail, one such metabolite that is frequently produced in LAB fermentations in amounts exceeding 1 % is lactic acid. Recently, it was demonstrated that lactic acid (or lactate) dose-dependently inhibits the release of pro-inflammatory cytokines by TLR-activated bone marrow-derived macrophages and dendritic cells. Lactate also modifies the redox state by lowering the intestinal enterocytes' reactive oxygen species levels. On the contrary, most other products derived from microbes that are produced during fermentation strictly depend on the strain. For example, bacteria found in plants and dairy products are known to synthetize different non-vitamin precursors to produce the B vitamins, which include folate, riboflavin, and B12 (Marco et al. 2017). Fermented foods containing appreciable levels of fermentation-produced organic acids (>100 mM), coupled with low water activity, salt, nitrite, and other antimicrobials, are solidly considered as safe (Adams and Mitchell 2002).

1.7 Fermented dairy products

Fermented milks can be divided into two main categories according to the predominantly present microorganisms. LAB species are the main source of lactic fermentations, which can be thermophilic (yogurt, bulgarian buttermilk), probiotic (acidophilus milk, yakult, bifidus milk), or mesophilic (natural fermented milk, cultured milk, cultured cream, cultured buttermilk). Another type of lactic fermentation is fungal-lactic fermentations, in which yeast species and LAB work together to produce alcoholic milks (kefir, koumiss, acidophilus-yeast milk), as well as moldy milks (Anagnostopoulos and Tsaltas 2019).

1.7.1 Yogurt

Yogurt is a fermented milk product produced by thermophilic lactic acid bacteria, primarily *Streptococcus thermophilus* and *Lactobacillus delbruec*kii spp. However, *Lactobacillus delbruec*kii subsp. *bulgaricus* is preferably consumed due to its renowned health beneficial impacts. The primary processes involved in making yogurt are the clarifying of milk and the subsequent separation of it into cream and skim milk, homogenization, pasteurization, inoculation of starter culture, incubation at 43 °C for 4 – 6 hours and swift cooling when the pH hits 4.0 – 4.7. Lactic acid, which is produced during fermentation, causes casein micelle strands to aggregate and form a gel matrix when the pH reaches about 4.6 (Arab et al. 2023), and parameter such as the viscosity and hardness in the final product, can changes varying the manufacturing conditions (Sumi et al. 2023).

1.7.2 Kefir

Kefir is a low-alcohol fermented mildly acidic milk, produced by fermentation of milk by lactic acid bacteria and yeasts at room temperature. The term is derived from the word *kef*, which means "pleasant taste" in Turkish. During fermentation, milk is fermented by several bacteria and yeasts, including *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., and *Acetobacter* sp. The type of milk and the diverse microbiological population from different culture types (kefir grain and commercial starter culture) affect the composition of kefir (Arslan 2015). Kefir grains are white to yellowish-white, cauliflower-like "florets" composed of an inert polysaccharide/protein matrix that contains a stable and specific microbial community of various lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeast species in a complex symbiotic relationship (Leite et al. 2012). Kefir grains arise from self-aggregation of *Lactobacillus kefiranofaciens* and *Saccharomyces* spp. to form tiny granules. Subsequently, the surface of the granules is colonized by the bacterium *Lentilactobacillus kefiri*, which forms the grains by aggregating with other microorganisms and milk components (González-Orozco et al. 2022). Particularly in kefir grains, the significant components are mostly LAB (*Streptococcus* sp., *Lactobacillus* sp., *Lactococcus* sp., and *Leuconostoc* sp.) and yeasts (*Candida, Kluyveromyces, Saccharomyces*, and *Pichia*) (Saleem et al. 2023).

1.7.3 Dahi

Dahi is a Asian traditional yogurt-like fermented milk, made by inoculating boiled milk with the previous dahi sample and letting it ferment naturally for two to three days at room temperature (Shangpliang et al. 2017).

About the microflora of LAB in traditional dahi fermented milk, it has been described that *Streptococcus bovis* is the dominant LAB species but relatively high frequency of *Limosilactobacillus fermentum, Lactobacillus delbrueckii* ssp. *bulgaricus, Enterococcus faecium* were found, while *Streptococcus thermophilus, Lactobacillus delbrueckii* ssp. *lactis, Leuconostoc mesenteroides* ssp. *mesenteroides, Leuconostoc mesenteroides* ssp. *dextranicum, Lactococcus lactis* ssp. *lactis, Lactococcus raffinolactis,* and *Pediococcus pentosaceus* were identified at lower frequency (Harun-ur-Rashid et al. 2007).

1.7.4 Koumiss

Koumiss is a fermented milk product prepared from mare's milk, famous in Asia, Russia, and Eastern Europe. Traditional methods of making koumiss using natural fermentation include the following steps: milk is poured into a wooden barrel, leather bag or plastic barrel that contains some koumiss residue as a fermentation starter; next, a wooden stick is used to stir the fresh mare milk vigorously to form a mixture that then ferments naturally at ambient temperature for 1 - 3 days. The yeast, lactic acid bacteria, other microorganisms and alcohol content are determined by fermentation time (Xue et al. 2023). In particular, the most commonly found bacterial species during koumiss fermentation include *Lactobacillus helveticus, Lactobacillus kefiranofaciens Lactococcus lactis, Lactococcus raffinolactis,* and *Citrobacter freundii* (Tang et al. 2020). Other research reveal the presence of *Lactobacillus helveticus, Streptococcus parauberis, Acetobacter pasteurianus, Lactobacillus kefiri, Enterococcus durans, Leuconostoc mesenteroides,* and *Acetobacter oryzoeni* (Bao et al. 2019; Zhao et al. 2024). With regard to yeasts, the species found in koumiss were *Dekkera anomala, Kazachstania unispora, Meyerozyma caribbica*, *Pichia*, and *Kluyveromyces marxianus* (Tang et al. 2020). During the koumiss fermentation, a progressive decrease of pH occurs, as consequence of lactose fermentation, with production of lactic, acetic and butyric acid. This process is strictly related to metabolic activity of *Acetobacter, Leuconostoc, Pediococcus,* and *Bacteroides* (Bao et al. 2019).

1.7.5 Buttermilk

The terms “traditional/conventional” and “cultured” can be used to describe whether the buttermilk is fermented spontaneously or by starter, while the processing conditions can influence the final product and obtain sour-cream or sweet-cream buttermilk (Antunes et al. 2009). Traditional buttermilk is the term referring to the liquid part resulting from butter making from fermented cream or milk, which has been fermented by naturally occurring lactic acid bacteria (LAB). On the other hand, cultured buttermilk is a commercial product obtained by the fermentation of

pasteurized skim milk, by a starter culture, consisting of mixed strains of *Lactococcus lactis* subspecies *cremoris* and *lactis,* and *Leuconostoc mesenteroides* spp. *cremoris* (Gebreselassie et al. 2016).

1.8 Fermented plant-based products

Consumer interest in milk substitutes made from nuts, legumes, seeds, pseudocereals, and cereals, has gained great attention during the past years for a number of diverse reasons. Primarily, lactose intolerance and allergies to milk proteins are two factors that limit the assumption of milk products. Additional frequent causes include dietary shifts away from animal-based diets and avoiding the greenhouse gas emissions from livestock, greatly impacting on the environment (Tangyu et al. 2019; Huang et al. 2023). Although plant-based products are touted as being less harmful to the environment, there is ongoing discussion and research regarding the sustainability benefits of these products. There is no doubt about the reduced environmental impact of producing legumes, but the contribution of legumes to the reduction of greenhouse gas emissions also depends on how the agro-ecosystem is managed (e.g., monocropping versus conservation agriculture). Furthermore, post-harvest processing could contribute to sustainability (Alcorta et al. 2021). Unfortunately, plant-based milk alternatives are often nutritionally unbalanced and their flavour profiles limit their acceptance. Fermentation can aid in improving the sensory profiles, nutritional qualities, texture and microbiological safety of plant-based milk substitutes in order to produce more valued and palatable products, without the addition of extra ingredients such as chemical additives. The relevant plant alternatives sources to produce non-dairy milk can be divided. Given the growing consumers' demand for healthier products, many brands have commercialized plant-based beverages, principally soy and oat milk derived from legumes and cereals, respectively (Mishra et al. 2023). However, legumes provide more balanced food options than the cereal grains (Affrifah et al. 2023) and were a predominant source of superior-quality plant protein (Qin et al. 2022).

1.8.1 Cereal-based beverages

Cereals are the edible grains or seeds that belong to the Gramineae family. In particular, wheat and rice are the most important crops globally, accounting for more than 50 % of all cereals produced worldwide (Jindal et al. 2023). Traditional beverages made from cereal are available in a large variety worldwide and are primarily consumed in Africa. These products are made from maize, millet, and/or sorghum by spontaneous fermentation from mixed microbial cultures involving yeasts and lactic acid bacteria. Some examples for African fermented beverages include Ben-saalga (Burkina Faso), Kanun-zak (Nigeria), Koko (Ghana), Mageu (Southern Africa and Arabian

Gulf countries), Munkoyo (Zamibia and Congo), Thobwa (Malawi and Tanzania), Uji (Uganda, Kenya and Tanzania), Bushera (Uganda). Other examples of cereal-based beverages can be found from the Balkan region (Bulgaria, Romania, Albania and Turkey), such as Boza, which is prepared from wheat, rye, millet or other cereals mixed with water and sugar (Angelov et al. 2018), as well as from eastern European countries, such as Kvass, which is produced from fermented rye and barley malt, rye flour and stale rye bread (Basinskiene et al. 2016). Several microorganisms are involved in cereal plant fermentations and mainly belong to the genera *Lactobacillus*, *Pediococcus*, *Bifidobacterium*, *Candida*, *Debaryomyces, Endomycopsis, Hansenula*, *Pichia*, *Saccharomyces*, and *Trichosporon* (Gotcheva et al. 2000; Mårtensson et al. 2000; Angelov et al. 2018; Patra et al. 2023).

1.8.2 Soybean

Glycine max, a legume belonging to the Fabaceae family (Leguminosae), was domesticated from its wild progenitor *Glycine soja* approximately 3000 – 5000 years ago in East Asia (Zhu et al. 2023). Significant morphological and physiological changes, such as the loss of pod dehiscence, occurred in cultivated soybean during domestication; this change is believed to have been crucial for the domestication of cultivated soybean (Li and Olsen 2016). At least twenty-five wild perennial species belong to the subgenus *Glycine*, widely distributed in Australia, Asia and parts of Africa. It is being cultivated in a variety of soil types, but the crop grows best in well-drained soil that is high in organic matter has a slight acidity (pH of 6.0 – 6.5) (Edde 2022), while its optimal growth temperature is between 20 and 30 °C (Lombardo et al. 2011).

Noteworthy, significant variations in nutritional characteristics i.e. protein, oil, fatty acids, soluble sugars and isoflavones contents among Chinese and the USA soybean cultivars was observed. The Chinese cultivars displayed higher levels of protein, oleic acid, and saturated fatty acids, while the USA cultivars displayed higher levels of oil, unsaturated fatty acids, sucrose, total soluble sugar, and total isoflavone. Furthermore, the main differentiating factors among the soybean cultivars were found to be protein, oil, oleic acid, linoleic acid, and isoflavone (Azam et al. 2021). Soybeans are widely acknowledged as a crop with significant economic value on a global scale and they are a primary source of exceptional plant protein, thanks to their fat, protein, carbohydrate and crude fiber content that is comparable to that of meat products. Although animal protein has long been considered the main source of dietary protein, in the recent years took place a paradigm shift in dietary consumption patterns, prompting the population towards plant-based food protein (Qin et al. 2022). Many studies on the nutritional value of soy highlight its high protein content. In the developing cotyledons, particles known as “protein bodies” or aleurone grains accumulate at least

60 – 70 % of the total protein in soybeans and about the 90 % of the seed protein is composed of globulins (Sasaki and Nunomura 2003). The main distinction between the proteins is the molecular size: about 70 % of total globulins are composed of the 7S and 11S fractions while the remaining minor constituents are 2S (15 %) and 15S (9.1 %) fractions. In detail, the 7S fraction consists of β- and γ-conglycinin (35 %), while the 11S fraction consists of glycinin (41.8 % of the total globulins). Soy products possess not only the attributes listed above, but also some other beneficial molecules, including phytosterols, bioactive peptides, isoflavones, saponins and thus, health benefits of these functional compounds generally lead to their use as dietary supplements (Qiao et al. 2022). However some soy products contain undesirable anti-nutrients like phytic acid, trypsin inhibitors, urease, and proanthocyanidins (Cai et al. 2021), that are metabolites than naturally occurring and that the plant produces to protect itself from pest attacks. To avoid that, several strategies were applied in order to reduce food levels of anti-nutrients including physical, chemical and biological process (Avilés-Gaxiola et al. 2018). Particularly, fermentation has been identified as a biological process that enhances the nutritional and organoleptic properties of soy products, since soy composition per se promotes the growth and incorporation of different microorganisms (Gao et al. 2013; Cai et al. 2021), thus representing a useful tool to reduce bacterial contamination of foods (Samtiya et al. 2020) and enhance its functional benefits (do Prado et al. 2022).

1.8.3 Fermented soymilk

Soymilk is a nutritious beverage obtained from heat-treated aqueous extract of homogenized previously soaked soybeans. Additionally, soymilk can turn to a yogurt-like paste following lactic fermentation (Chen 2013; Fukuda et al. 2017). Soy-based beverages represent the dominant plant-based milk alternatives in the Western world, due to its nutritional composition (Tangyu et al. 2019), with high biological value proteins of vegetal origin and low fat content, free from lactose and cholesterol, and rich in bioactive compounds (de la Bastida et al. 2023). Besides other benefits are its high digestibility and low cost (Sethi et al. 2016).

Nevertheless, soymilk contains aldehydes such as pentanal and hexanal that exacerbate an unpleasant or “beany” aftertaste, which are formed mainly by the hydro-peroxidation of polyunsaturated fatty acids catalyzed by lipoxygenase, that considerably limit consumer acceptance (Abdelghani et al. 2022). However, fermentation of soymilk by lactic acid bacteria improves its physicochemical and organoleptic properties as well as its nutritional value (Zong et al. 2022), by reducing the beany flavor and increasing product acidity. The pH decrease can protect the product from developing spoilage microorganisms and consequently increase its shelf life, not changing the sensory or technological characteristics of the product (Abdelghani et al. 2022). To

date, plant-based milk fermentation mainly uses mono-cultures of microbes, such as lactic acid bacteria, bacilli, and yeasts (Tangyu et al. 2019). Many different bacteria were described as starter for lactic acid fermentation of soymilk, including *Bifidobacterium, Lactobacillus,* and *Enterobacterium* (Fukuda et al. 2017). The use of specific starter directly influence the variations of the beneficial properties of the fermented product (Hossain 2022).

1.8.4 Tofu

Soymilk, through a coagulation process, can be used to produce tofu, which is a traditional oriental food, composed principally by proteins, polyphenols and lipids, that in the recent years has gained great commercial relevance because of its employment in vegan-based diets. Tofu is a highly nutritional, gel-like soy food, obtained by soaking and grinding of soybeans in water, filtering, boiling to obtain soymilk, that afterward it is coagulated and molded (Prabhakaran et al. 2006). There are three fundamental steps in tofu preparation, resulting in specific characteristics of the product type: i) soymilk extraction and solid content, ii) coagulation method (types of coagulants, breaking or not after curd formation), and iii) pressing or not (Chang and Hou 2003). In particular, coagulation may be obtained by several different types of coagulants, which are divided in acids, enzymes, and salts. The choice and addition of coagulants is crucial to the formation of water-based protein gel and also it influences the yield, taste, texture, and digestive properties of tofu (Shi et al. 2024). The acid coagulation methods (such as citric acid, lactic acid, acetic acid, white vinegar, and lemon juice) cause gelation of soy proteins by isoelectric precipitation, while enzyme coagulants, such as transglutaminase, can assemble proteins through isopeptide bonds among the amine group of glutamine residue and the ξ-amino group in the lysine residue. Finally, with salts (e.g. calcium sulphate, calcium chloride, calcium acetate, calcium lactate, and magnesium chloride), the formation of a three-dimensional network structure occurs because of salt bridges cross-connecting proteins (Zeppa et al. 2021). In particular, to this latter group, belong the nigari salts coagulants such as natural nigari, refined nigari, calcium chloride, and seawater. Natural nigari is extracted from seawater by removing most or all of the table salt (NaCl) and water and it is made primarily of $MgCl_2 \cdot 6H_2O$ (92.3 %) plus all of the other salts and trace minerals in seawater (3.8 % $MgSO_4$, 1.7 % NaCl, 1.2 % KCl, and 1 % $CaSO_4$). On the contrary, refined nigari is a relatively pure magnesium chloride (99.5 %). Finally, calcium chloride ($CaCl_2$) is not found in seawater, so it is not a traditional nigari-type coagulant (Chang and Hou 2003). Recently, a large body of research focused on approaches to improve the texture of tofu products. In tofu making, there are some hydrocolloids used as gelling agents or stabilizers, such as carrageenan, chitosan, guar gum, and gum arabic, to improve texture, water retention or extend shelf life (Chen et al.

2023). Moreover, the use of lactic acid bacteria to produce soymilk curds has been evaluated, as well as new processes to produce fermented tofu. In fact, soymilk fermentation with selected LAB strains and subsequent protein precipitation has been demonstrated to prevent or delay undesired microbial and chemical spoilage, improve the stability and the quality of the final product (Serrazanetti et al. 2013).

1.9 Functional proprieties of fermented soy products

Fermented soy-based foods offer a high content of health-promoting compounds that offer a high nutritional index and positive effects on human health. Of note, the bioavailability of these compounds can be modified during fermentation. In fact, the correct fermentation strategy and processing techniques alter their content. Therefore, understanding the most suitable parameters for fermentation is a pivotal milestone, which would lead to improved nutritional value and functional benefits of fermented soy products (Qiao et al. 2022). The soybean is processed to make soymilk and tofu (soy curd), and it is fermented to produce also a variety of ethnic fermented soybean products, including tempe, chungkokjang, miso, shoyu, natto, kinema, and thua nau (Anagnostopoulos and Tsaltas 2019).

Soybean product contain specific antioxidative components that can be enhanced during fermentation, mainly attributable to phenolic compounds which are insoluble esters bound to cell walls, proteins, lipids or polysaccharides (Qiao et al. 2022). Proteases, amylases, and glucosidase are just some of the microbial enzymes that degrade polymers during fermentation and result in the changes in the composition of fermented soybean (Shahidi and Yeo 2016; Padmavathi et al. 2018). The predominant phenolic components found in soy-derived products are flavonoids, a broad group of plant-based polyphenols with a C_6-C_3-C_6 three-ring core, which main subclasses are flavanones, flavan-3-ols, flavonols, isoflavonoids, anthocyanins, and anthocyanidins (Charlton et al. 2023). In detail, isoflavones are presents in their glucoside (genistin, daidzin, and glycitin) or aglycone (genistein, daidzein, and glycitein) forms, while the main phenolic acids are syringic, chlorogenic, gallic, vanillic, and ferulic acids (Rodríguez-Roque et al. 2013).

The chemical structure of flavonoids plays a fundamental role in the antioxidant activity. Specific hydroxylation pattern, in the ring B of the flavonoid molecules and/or a C2═C3 double bond in conjugation with a C4-carbonyl group, methoxyl groups and O3–H groups increases their antioxidant properties while the presence of saccharide groups reduces their antioxidant activity (Vo et al. 2019). The radical scavenging activity of flavonoids is achieved through two mechanisms in particular, namely Single Electron Transfer (SET) and Hydrogen Atom Transfer (HAT). In detail, by the HAT mechanism the antioxidant reacts with the free radical transferring

one of its hydrogen atoms to the radical via homolytic rupture of an -OH bond; while in the SET mechanism, a single electron is transferred from the antioxidant to the radical which lead to the ionization of both compounds along the process (de Souza Farias et al. 2021; Charlton et al. 2023). Isoflavones, isomers of flavones with the B-ring in the 3-position instead of the 2-position of the C ring, exhibit the same radical scavenging activity to the corresponding flavones with regards to the same hydroxyl group position and sugar moiety, demonstrating that the position of the B-ring is relatively negligible in terms of antioxidant activity (Charlton et al. 2023).

Fermented soy, also contains other classes of useful functionals molecules like conjugated linolenic acid, long-chain omega-3, -6, and -9 polyunsaturated fatty acids, and bioactive peptides, which are typically present in glycosylated, esterified, thiolated or hydroxylated form (Fukuda et al. 2017). Fermentation of soy products significantly improve their antioxidant capacity, increase levels of vitamin B2, vitamin B12, saponins, lecithin, and γ-Aminobutyric acid (GABA). In particular, GABA a non-protein amino acid involved in several metabolic functions, including the central nervous system regulation, blood pressure reduction, hormone secretion and regulation hormone secretion (Tsai et al. 2010; Jayachandran and Xu 2019; Wu et al. 2020; Zhuang et al. 2023). Moreover it has been reported that extract of fermented soy bean product showed a preventive effect on lipid peroxidation in the liver and of cholesterol-fed rats, through enhancing the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) (Wang et al. 2008).

1.10 Lactiplantibacillus plantarum

Recently, it has been proposed a reclassification based on polyphasic approach of the genus *Lactobacillus* into 25 genera including the emended genus *Lactobacillus*, which includes host-adapted organisms that have been referred to as the *Lactobacillus delbrueckii* group, *Paralactobacillus* and 23 novel genera. For these 23 novel genera the following name have been designed: *Holzapfelia*, *Amylolactobacillus*, *Bombilactobacillus*, *Companilactobacillus*, *Lapidilactobacillus*, *Agrilactobacillus*, *Schleiferilactobacillus*, *Loigolactobacilus*, *Lacticaseibacillus*, *Latilactobacillus*, *Dellaglioa*, *Liquorilactobacillus*, *Ligilactobacillus*, *Lactiplantibacillus*, *Furfurilactobacillus*, *Paucilactobacillus*, *Limosilactobacillus*, *Fructilactobacillus*, *Acetilactobacillus*, *Apilactobacillus*, *Levilactobacillus*, *Secundilactobacillus*, and *Lentilactobacillus* (Zheng et al. 2020).

Lactiplantibacillus plantarum (*Lp. plantarum*), formerly known as *Lactobacillus plantarum*, is an interesting probiotic candidate with a long history in the food industry as starter cultures in the production of a wide variety of fermented foods (Fidanza et al. 2021; Garcia-Gonzalez et al. 2021).

Lp. plantarum is a promising LAB species that is widely used as a probiotic and starter culture in the food industry, considered Qualified Presumption of Safety (QPS) from the European Food Safety Authorities (EFSA) and listed as "generally recognized as safe" (GRAS) by the United States Food and Drug Administration (FDA). It presents a genome of 3.3 Mb which is greater than others LAB (2 – 2.7 Mb), this reflects resembles improved metabolic flexibility (Yilmaz et al. 2022). Regarding its metabolism, *Lp. plantarum* is able to ferment hexoses into to lactic acid via the Embden–Meyerhof–Parnas pathway, while pentoses are converted to lactic and acetic acids via the 6-phosphogluconate/phosphoketolase pathway, which makes them heterofermentative lactobacilli (Corsetti and Valmorri 2011). Several sugars can be used as carbon and energy sources. Intracellular uptake and concomitant phosphorylation of sugars is mediated by phosphoenolpyruvate–phosphotransferase (PEP–PET) sugar transport systems (Deutscher et al. 2006; Corsetti and Valmorri 2011).

A recent comparative study among several *Lp. plantarum* strains, pointed out the differences in specific functional genes involved in metabolization of carbohydrates and oligosaccharides, in the biosynthesis of short-chain fatty acid (SCFAs), acetoin, vitamin, GABA, antioxidant, as well as the biosynthesis of the non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), antibiotic resistance and, finally, in the alcoholic degradation activity (Choi et al. 2021). Noteworthy, *Lp. plantarum* is a notable microorganism that has gained a lot of attention due to its potential to produce EPS. The capacity of *Lp. plantarum* strains to produce exopolysaccharides (EPSs) has been widely employed in food processing and preservation, and it greatly enhances the quality and functionality of food. In fact, studies concerning the use of *Lp. plantarum* as a starter have demonstrated its technological ability to ferment food and beverages. Besides, it has been suggested that the use of *Lp. plantarum* as starter for fermentation of plant-based beverages, can modify their rheological proprieties specifically correlating this effect to EPS production (Li et al. 2014). Furthermore, it is reported that *Lp. plantarum* EPSs demonstrated a range of bioactivities, including immunomodulation, anticancer, antioxidation, biocompatibility and prevention of the formation of biofilms from pathogenic bacteria (Jiang and Yang 2018). In its regard, the study of anti-inflammatory effect of the fermented plant-based products, have demonstrated that they lead to a decreased content of inflammatory cytokines in mice serum and, consequently, to the amelioration of colitis (Sun et al. 2022).

Finally, certain *Lp. plantarum* strains can product bacteriocins, that are natural antibacterial peptides ribosomally synthetized. These molecules with antibiotic biological activity, have no antibacterial effect on the strains that produce them, but they can inhibit or kill both phylogenetically related and unrelated microorganisms. Of note, bacteriocins are generally

recognized as safe, with many commercially available as approved food preservatives (Onyeaka and Nwabor 2022) and thus have received a lot of attention because they may replace chemical preservatives (Wang et al. 2023). In detail, *Lp. plantarum* produces a bacteriocin known as "plantaricin", belonging to class I (lantibiotic) and class II (non-lantibiotic) bacteriocins. Mostly these molecules are non-lantibiotic, small (<10 kDa) two-peptide molecules, hydrophobic, cationic, unmodified and stable to heat. Great interest arises by these plantaricins since several studies demonstrated they help to improve the functioning of the human digestive system, which in turn boosts the immune system through the production of extracellular proteins and exopolysaccharides (Aziz et al. 2022; Yilmaz et al. 2022).

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Chapter 2

Preliminary screening of *Lactiplantibacillus plantarum*

2.1 Introduction

Besides the literal meaning, some of the criteria for microorganisms selection must be considered before their applicability as probiotics in the human diet: a probiotic agent must show non-pathogenic properties; ability to survive in the digestive tract; adherence to the intestinal epithelium; colonization of the intestinal tract; production of antimicrobial compounds; adequate survival (stability) in the form of powder, liquid or food (Shewale et al. 2014; Hill et al. 2014; EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) et al. 2018). In addition to these primary characteristics, there are further functional attributes that can be contemplated in the selection of new probiotics (Nataraj et al. 2020; Zendeboodi et al. 2020; Żółkiewicz et al. 2020; Peluzio et al. 2021). Specifically, the selection of lactic acid bacteria (LAB) as probiotics possessing β-glucosidase activity, and also capable of producing exopolysaccharides (EPSs) and γ-aminobutyric acid (GABA), has received much attention in recent years (Michlmayr and Kneifel 2014; Wang et al. 2015, 2021; Diez-Gutiérrez et al. 2020; Cui et al. 2020). GABA (IUPAC name: 4-aminobutanoic acid) is a non-protein ubiquitous amino acid that is biosynthesized via the α-decarboxylation of glutamate, by glutamate decarboxylase (GAD) (Sarasa et al. 2020). The pharmaceutical and food industries have been performing extensive studies to develop GABA-rich dietary supplements and fermented foods aiming to leverage the manifold health benefits of this amino acid, including gut modulation, neurostimulation, and cardioprotection (Quílez and Diana 2017; Diez-Gutiérrez et al. 2020). About the β-glucosidase activity of LABs, it is of particular interest since it is able to increase the bioavailability of polyphenols. Several studies indicate the potential effect of polyphenols such as flavonoids and anthocyanins in reducing the incidence of cardiovascular disease, cancer, hyperlipidemias, and other chronic diseases through the intake of anthocyanin-rich foods (Zhang et al. 2021). However, the effectiveness strongly depends on their bioavailability (Viskupičová et al. 2008). Anthocyanins contained in fruit and vegetables are bound to one or more glycosidic units. The biological activity of anthocyanins can be impaired by glycosylation, which increases their solubility in water, but on the other hand, it also makes them less reactive to free radicals and metals, thus reducing their antioxidant activity (de Pascual-Teresa and Sanchez-Ballesta 2008). The glycosidase activity of several LABs removes the glycoside and this hydrolysis increases the bioavailability and intestinal absorption of flavonoids, which is essential for their health benefits (Yuksekdag et al. 2017).

Moreover, several LABs are also capable of producing capsular or extracellular polysaccharides (EPS), with desirable technological properties and biological activities (Das et al. 2014; Wang et al. 2021). EPS are widely used as viscous agents, stabilizers, and emulsifiers in the food industry (Sanalibaba and Çakmak 2016; Zannini et al. 2016; Majee et al. 2017; Singh and Saini 2017). In addition, the EPS produced by LABs may be important for the survival of probiotics during gastrointestinal transit and adhesion to the intestinal wall, as well as being known to exhibit several prebiotic properties (Das et al. 2014; Caggianiello et al. 2016; Singh and Saini 2017).

Among LABs, *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) (Zheng et al. 2020) is a versatile bacterium with high adaptability to many different conditions, being isolated from several ecological niches including milk, fruit, cereal crops, vegetables, bee bread, and fresh meat (Siezen et al. 2010). As with other LABs, *Lp. plantarum* is a common host in the gastrointestinal tracts of insects, fish and mammals, including humans (Siezen et al. 2010; Iorizzo et al. 2021b, a). The metabolic characteristics that are intrinsic of *Lp. plantarum* are the reason for their versatility and success in industrial applications, not only as a starter culture but also as a probiotic (Liu et al. 2018; Goel et al. 2020; Garcia-Gonzalez et al. 2021). Furthermore, some strains of *Lp. plantarum* are known to produce various natural antimicrobial compounds (Arena et al. 2016; Tremonte et al. 2017), GABA and EPS (Di Cagno et al. 2010; Zhuang et al. 2018; Silva et al. 2019) and to exhibit antioxidant (Li et al. 2012; Das and Goyal 2015) and β-glucosidase activity (Sirilun et al. 2012; Iorizzo et al. 2016). *Lp. plantarum* has the Qualified Presumption of Safety (QPS) status from the European Food Safety Authority (EFSA) and Generally Recognized As Safe (GRAS) status from the US Food and Drug Administration (US FDA) (EFSA Panel on Biological Hazards (BIOHAZ) et al. 2022). Moreover, they are microorganisms with a documented history of food use (Darby and Jones 2017; Seddik et al. 2017; Behera et al. 2018).

While a considerable number of well-characterized probiotic strains are available worldwide, research into novel strains with specific properties is still of major appeal. This is because even microorganisms within the same species show differential phenotypic characteristics (Ramos et al. 2013; Campana et al. 2017). On the basis of this scientific evidence, this work aims to evaluate some bio-functional properties of five *Lp. plantarum* strains, isolated from bee bread and partially characterized in previous studies (Iorizzo et al. 2020b, a). In addition to antimicrobial, antioxidant and β-glucosidase activities, the ability of these LABs to produce EPS and GABA were evaluated. The main objective was to evaluate a possible candidacy of these *Lp. plantarum* strains as dietary supplements in the human diet and/or as microbial cultures for the production of functional food.

2.2 Materials and Methods

2.2.1 Lactiplantibacillus plantarum cultures

In this investigation, five *Lp. plantarum* strains (LP8, LP25, LP86, LP95, and LP100) isolated from bee bread (Iorizzo et al. 2020a) and belonging to the Food Microbiology Culture Collection of the DiAAA (Department of Agricultural, Environmental and Food Sciences, University of Molise, Campobasso, Italy) were used. 16S rDNA sequences were submitted in GeneBank (Clark et al. 2016) under accession numbers from OM033651 to OM033655 (accessed on 28 December 2021). *Lp. plantarum* ATCC 14917, belonging to the American Type Culture Collection (ATCC Manassas, VA, USA), was used as reference strain with proven *in vitro* probiotic properties (Wang et al. 2018; Mantzourani et al. 2018; Li et al. 2018).

2.2.2 Antimicrobial activity

The antimicrobial activity of the *Lp. plantarum* strains (producers) was evaluated against the following indicator bacteria: *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 11775, *Proteus mirabilis* ATCC 29906, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853, belonging to the American Type Culture Collection (ATCC Manassas, VA, USA). The LABs and the indicator strains were grown at 37 °C in MRS and BHI broth (Oxoid Ltd., Hampshire, UK), respectively.

The *Lp. plantarum* strains in log phase (10^8 CFU/mL) were centrifugated (8,000 rpm for 10 min at 4 °C) and filtered (cellulose acetate membrane, pore size 0.22 μm Sigma-Aldrich, Merck KGaA, Darmstadt, DE) to obtain the cell-free supernatant (CFS). The antimicrobial activity of CFS was evaluated according to Iorizzo et al. (2020b) with some modifications. In brief, 20 mL of BHI soft agar (0.7 % agar, *w*/*v*) inoculated with an overnight culture of indicator bacteria (final concentration of about 10^7 CFU/mL) were poured into Petri plates (Ø 90 mm). Wells of 5.0 mm in diameter were bored into the single plate and 50 μL of CFS, of each producer strain, were placed into different wells. As a control, 50 μL of MRS adjusted to pH 3.8 with hydrochloric acid 1 N (Sigma-Aldrich, Merck KGaA, Darmstadt, DE) was used. After incubation at 37 °C for 48 – 72 h, the plates were observed and antibacterial activity was reported as the diameter (mm) of the clear zone of inhibition (ZOI) around the inoculated wells (Testa et al. 2019).

2.2.3 Cell protein assay

For subsequent analysis (EPS and GABA production; antioxidant activity) the LAB cultures (10^8 CFU/mL), grown in the specific media reported below, were centrifugated (8,000 rpm for 10 min

at 4 °C) to obtain the cell pellet (CP). The CP was washed twice in sterile saline solution (NaCl 0.9 %, *w*/*v*), resuspended in NaOH 0.1 M according to Iorizzo et al. (2022), and then subjected to three cycles of sonication (Labsonic M; Sartorius Lab Instruments GmbH & Co. KG, Goettingen, DE) at 12 W for 30 s, with 60 s of pause between the cycles to improve cellular lysis. Subsequently, the samples were centrifugated at 13,000 rpm for 5 min at 4 °C, and cell extract supernatants (CES) were used for protein quantification according to Di Martino et al. (2020) using a BioSpectrometer (Eppendorf, Hamburg, DE). Total protein concentrations were calculated by mean of a calibration curve using Bovine Serum Albumin (BSA) as standard. All chemicals used were supplied by Sigma-Aldrich, Merck KGaA, Darmstadt, DE.

2.2.4 β-glucosidase activity

For the assessment of β-glucosidase activity, the tested LABs were cultured in modified MRS (MRS_m) according to Ávila et al. (2009). The bacterial cultures (10 mL, 10^8 CFU/mL) were centrifugated (8,000 rpm for 10 min at 4 °C) to obtain the cell pellet (CP_{GA}); they were washed in sterile saline solution (NaCl 0.9 %, *w*/*v*) and resuspended in isotonic Phosphate Buffer Saline (PBS) pH 7.4. The β-glucosidase activity of the *Lp. plantarum* strains was performed as described by Ávila et al. (2009), with some modification. Briefly, β-glucosidase reactions were performed mixing 50 µL of 20 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) in PBS pH 6.5, with 50 µL of bacterial suspension (OD_{600} 0.5) and bringing the final volume of the solution to 1 mL with PBS. Next, 200 µL of the mix were placed in a microplate well and read at 405 nm in kinetic mode with a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific, Waltham, MA, USA) for six hours. Enzyme activity was expressed as the amount of β-glucosidase that released 1 nmol of *p*-nitrophenol (*p*-NP) per unit volume per minute: *p*-NP/(min mL). A calibration curve was obtained using *p*-nitrophenol as standard. The values of β-glucosidase activity were expressed as the ratio of enzyme units. All chemicals used were supplied by Sigma-Aldrich, Merck KGaA, Darmstadt, DE, and the tests were conducted in triplicate.

2.2.5 Exopolysaccharide (EPS) production

For EPS production, LABs were grown in MRS_m. Over 48 h at 37 °C, the cultures (10^8 CFU/mL) were centrifugated (8,000 rpm for 10 min at 4 °C) to obtain the cell-pellet (CP_{EPS}) and the cell-free supernatant (CFS_{EPS}).

The CP_{EPS} and CFS_{EPS} were used for the determination of EPS-b (bound exopolysaccharides) and EPS-r (released exopolysaccharides), respectively, as described by Tallon et al. (2003). EPS quantification was performed by the anthrone-sulfuric acid colorimetric assay as reported by

Laurentin and Edwards (2003). The culture medium without bacterial inoculum was used as control. The EPS-r values are expressed as μmol glucose Eq./mL, while the absolute concentrations of EPS-b* are expressed as nmol glucose Eq./mL. Instead, EPS-b values related to cellular protein content (EPS-b**) were expressed as the ratio between nmol glucose Eq. and cell proteins (μg BSA Eq.). The cell proteins were determined as described in section 2.2.3. The tests were conducted in triplicate. All chemicals used were supplied by Sigma-Aldrich, Merck KGaA, Darmstadt, DE.

2.2.6 Antioxidant activity (ABTS assay)

LAB cultures (10 mL) in MRS_m, over 48 h at 37 °C, were centrifugated (8,000 rpm for 10 min at 4 °C) at their log phase (10^8 CFU/mL) to obtain the cell pellet (CP_{AA}). The CP_{AA} were washed twice in sterile saline (NaCl 0.9 %, *w*/*v*), resuspended in cold pure methanol (1 mL) and subjected to three cycles of sonication (Labsonic M; Sartorius Lab Instruments GmbH & Co. KG, Goettingen, DE) at 12 W for 30 s, with 60 s of pause between the cycles to improve cellular lysis. Subsequently, the samples were centrifugated at 13,000 rpm for 5 min at 4 °C and cell extract supernatants (CES_{AA}) were used to evaluate total antioxidant activity (TAA), using the 2,2 azino-bis 3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) radical cation assay according to Re et al. (1999), with some modifications. Briefly, ABTS was dissolved in pure methanol to a concentration of 7 mM. ABTS radical cations ($ABTS^{\bullet+}$) were produced by reacting the ABTS methanol solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 24 h before use. The $ABTS^{\bullet+}$ solution was diluted with cold pure methanol to an optical density (OD) of 0.700 at 745 nm. Then, 100 μL of CES_{AA} were mixed with 900 μL of the $ABTS^{\bullet+}$ solution. The OD was measured at 745 nm after 6 min in the dark at room temperature using a BioSpectrometer (Eppendorf, Hamburg, DE). Trolox was used as the standard for the calibration curve.

The antioxidant activity was expressed as the ratio (*w*/*w*) between μg/mL Trolox and mg/mL of cell proteins (BSA Eq.) (Zambrowicz et al. 2015). The cell proteins were determined as described in section 2.2.3. All the chemical compounds used were supplied by Sigma-Aldrich, Merck KGaA Darmstadt, DE, and the tests were conducted in triplicate.

2.2.7 γ-aminobutyric acid (GABA) production

For the GABA production assay, the *Lp. plantarum* strains were grown at 37 °C in LFM medium, whose composition is pyridoxal-5'-phosphate (40 mg/L), D-glucose (20 g/L), L-cysteine (100 μM), L-phenylalanine (100 μM), L-histidine (100 μM), L-glycine (100 μM), L-glutamic acid

(100 µM). The broth cultures (10 mL; 10^6 CFU/mL) were centrifugated to obtain the cell pellet (CP_{LFM}). The CP_{LFM} were washed twice in sterile saline solution (NaCl 0.9 %, *w*/*v*), resuspended in 600 µL of ethanol/water (40/60, *v*/*v*), and then subjected to three cycles of sonication (Labsonic M; Sartorius Lab Instruments GmbH & Co. KG, Goettingen, DE) at 12 W for 30 s, with 60 s of pause between the cycles to improve cellular lysis. Subsequently, the samples were centrifugated at 13,000 rpm for 5 min at 4 °C and cell extract supernatants (CES_{LFM}) were used for GABA quantification via High Performance Liquid Chromatography (HPLC), using a Hewlett Packard 1100 (Agilent, Santa Clara, CA, USA) equipped with Degasser G1322A, Binary Pump G1312A, Autosampler ALS G1313A, Thermostatted Column Compartment G1316A, DAD Detector G1315B and Fluorescence Detector G1321A. For the automatic pre-column derivatization, 20 µL of CES_{LFM} were added to 40 µL of *o*-Phthalaldehyde (OPA) reagent (Agilent, Santa Clara, CA, USA). Subsequently, 30 µL of OPA-derivatives mix were injected into a reverse-phase column (ZORBAX Eclipse Plus C18, 4.6 × 250 mm 5 µm; Agilent, Santa Clara, CA, USA), protected with a SecurityGuard Phenomenex (Torrance, CA, USA). The elution was performed at a flow rate of 0.85 mL/min, at 27 °C, using a solution of sodium acetate buffer 50 mM + tetrahydrofuran 0.3 % + methanol 20 % pH 6.4 and methanol 100 %, as described by Carillo et al. (2019). The fluorescence of OPA derivatives was detected at 340 nm excitation and 450 nm emission wavelengths. The GABA identification and quantification were carried out using HP ChemStation software ver. A.06.03 (Agilent, Santa Clara, CA, USA), compared to retention times and internal peak areas of reference standards. The data were expressed as ratio between pmol of GABA and cell proteins content (µg BSA Eq.). The cell proteins were determined as described in section 2.2.3. All chemicals standards used were supplied by Sigma-Aldrich, Merck KGaA, Darmstadt, DE, and the tests were conducted in triplicate.

2.2.8 Qualitative screening of biogenic amines production

The ability to produce different biogenic amines was analyzed according to method described by Bover-Cid and Holzapfel (1999). Briefly, LABs were subcultured 5 times in MRS broth supplemented with 0.1 % of each precursor amino acid (all from Merck, Darmstadt, Germany), comprising tyrosine free base, histidine monohydrochloride, ornithine monohydrochloride and lysine monohydrochloride, in addition to supplementation with 0.005 % of pyridoxal-5-phosphate to promote the enzyme induction before biogenic amine screening. The decarboxilase medium (DM) was prepared with glucose (0.05 %), NaCl (0.25 %), $MgSO_4$ (0.02 %), $MnSO_4$ (0.005 %), $FeSO_4$ (0.004 %), ammonium citrate (0.2 %), Tween 80 (0.1 %), di-potassium phosphate (0.2 %) and calcium carbonate (0.01 %). Moreover, pyridoxal-5-phosphate (0.005 %) was included to the

decarboxylase medium as a cofactor for the decarboxylation reaction to enhance the amino acid decarboxylase activity. Finally the concentration of each amino acid was (tyrosine free base, histidine monohydrochloride, ornithine monohydrochloride, and lysine monohydrochloride) was 1 %. Bromocresol purple (0.006 %) was used as pH indicator, then 2 % agar was added. Finally, the pH was adjusted to 5.3 and the culture medium was autoclaved for 10 minutes at 121 ℃ to avoid excessive hydrolysis of the low pH agar. Decarboxylation of the amino acid to the corresponding biogenic amine was detected by the colour change of the medium to purple. All strains were streaked in triplicate on the decarboxylase medium plates with and without amino acids (as negative control), and were incubated for 4 days at 37 °C, under aerobic and anaerobic conditions.

2.2.9 Enzyme profile

The enzyme profile of *Lp. plantarum* LP95 strain was analyzed using the API ZYM Kit (BioMerieux, Lyon, FR) according to the manufacturer's instructions. In detail, the bacterial cells were harvested and resuspended in API Suspension Medium (2 ml), and then the bacteria suspension was adjusted to a turbidity of 5 – 6 McFarland. Subsequently, 65 µL of bacterial cells were inoculated in API ZYM strip and incubated at 37 °C for 4 h. After incubation reagents A and B were added for at least 5 minutes before screening of color changes.

2.2.10 Statistical analysis

Experiments were performed in triplicate (n = 3) and all data are expressed as the mean ± standard deviation (± SD). Statistical analysis was performed by analysis of variance (ANOVA) followed by Tuckey's multiple comparisons. Statistical significance was set to p-values < 0.05. The software SPSS Statistics 21 (IBM Corp, Armonk, NY, USA) was used for the analysis.

2.3 Results

2.3.1 Antimicrobial activity

The numerical data of the antimicrobial activity, estimated as clear zone of inhibition ZOI (mm), exhibited by LABs against various indicator bacteria are reported in Table 2.1. In Figure 2.1, the results are shown as inhibition rate (%). More specifically, no antimicrobial activity was observed when MRS pH 3.8 was used, while the six tested *Lp. plantarum* strains presented different inhibitory activities against the five indicator bacteria. The cell free supernatant (CFS) of all six *Lp. plantarum* strains inhibited the development of *P. aeruginosa* and *E. faecalis*. With the exception of LP25, the other LAB strains were effective against *E. coli* and *P. mirabilis*. The *Lp. plantarum* LP86, LP95, LP100 and ATCC 14917 strains were effective in counteracting the growth of *S. aureus*. The highest antagonistic activity of the tested *Lp. plantarum* strains was against *P. aeruginosa* with a ZOI between 18 (LP100) and 24 mm (LP8).

Table 2.1 Antimicrobial activity at 37 °C by cell-free supernatant (CFS) from the five *Lp. plantarum* strains against different indicator bacteria. The data (mean ± SD; n = 3) are expressed as zone of inhibition ZOI (mm).

Indicator bacteria	*Lp. plantarum* strains					
	LP8	**LP25**	**LP86**	**LP95**	**LP100**	**ATCC 14917**
P. aeruginosa ATCC 27853	24.0 ± 3.0 ^{a}	21.0 ± 1.0 ^{a}	20.0 ± 2.0 ^{a}	19.0 ± 2.0 ^{a}	18.0 ± 3.0 ^{a}	20.0 ± 2.0 ^{a}
E. coli ATCC 11775	9.0 ± 3.0 ^{a}	0.0 ± 0.0 ^{b}	9.0 ± 2.0 ^{a}	9.0 ± 1.0 ^{a}	10.0 ± 1.0 ^{a}	7.0 ± 3.0 ^{a}
P. mirabilis ATCC 29906	8.0 ± 2.0 ^{b}	0.0 ± 0.0 ^{c}	13.0 ± 2.0 ^{a}	12.0 ± 3.0 ^{a}	14.0 ± 1.0 ^{a}	9.0 ± 3.0 ^{a}
E. faecalis ATCC 29212	8.0 ± 1.0 ^{a}	9.0 ± 1.0 ^{a}	7.0 ± 2.0 ^{a}	10.0 ± 1.0 ^{a}	9.0 ± 3.0 ^{a}	7.0 ± 2.0 ^{a}
S. aureus ATCC 29213	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{c}	7.0 ± 1.0 ^{a}	8.0 ± 1.0 ^{a}	8.0 ± 2.0 ^{a}	4.0 ± 2.0 ^{b}

Different lowercase letters (a–c) in each row indicate significant differences ($p < 0.05$).

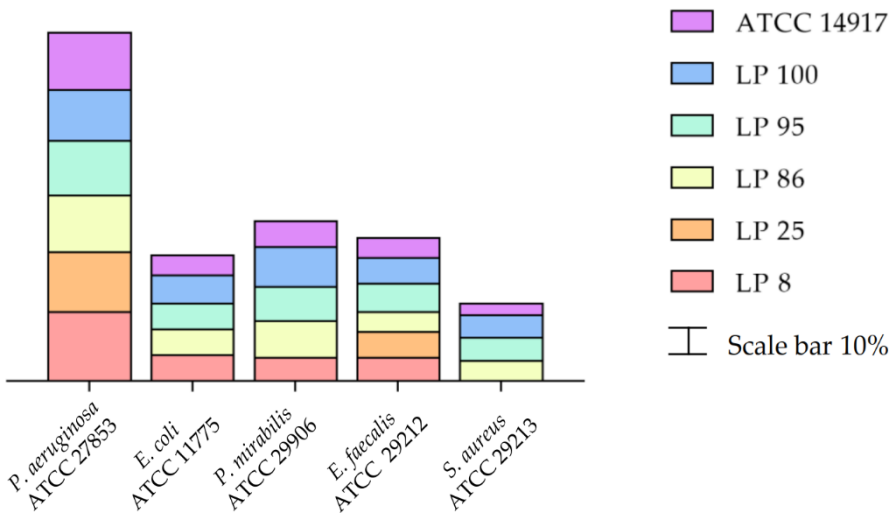

Figure 2.1 Antimicrobial activity of the *Lp. plantarum* strains CFS against different indicator bacteria expressed as inhibition rate (%).

2.3.2 EPS production

The values of EPS production were reported in Table 2.2. In detail, the EPS-r values ranged from 2.84 (LP25) to 3.43 μmol glucose Eq./mL (LP8), while the values of EPS-b* varied from 38.95 (LP86) to 223.42 nmol glucose Eq./mL (LP8). Lastly, about the EPS-b** content, the range of values was between a minimum of 0.016 (LP86) and a maximum of 0.069 (LP95) nmol glucose Eq./μg BSA Eq. (bovine serum albumin).

Table 2.2 EPS-released (EPS-r), EPS-bound (EPS-b) and EPS-bound related to cell proteins (EPS-b**). All values are expressed as mean ± standard deviation (n = 3).

	Lp. plantarum strains					
EPS fractions	**LP8**	**LP25**	**LP86**	**LP95**	**LP100**	**ATCC 14917**
EPS-r	3.43 ± 0.08 [a]	2.84 ± 0.15 [b]	3.29 ± 0.20 [a]	3.06 ± 0.17 [a]	3.03 ± 0.18 [a]	3.16 ± 0.13 [a]
EPS-b*	135.91 ± 5.67 [c]	136.23 ± 5.08 [c]	38.95 ± 1.91 [d]	223.42 ± 7.90 [a]	160.96 ± 7.94 [b]	51.45 ± 2.74 [d]
EPS-b**	0.048 ± 0.007 [b]	0.046 ± 0.005 [b]	0.016 ± 0.004 [c]	0.069 ± 0.011 [a]	0.050 ± 0.004 [b]	0.026 ± 0.006 [c]

Different lowercase letters (a–d) in each row indicate significant differences ($p < 0.05$).
The data are expressed as follows: EPS-r: μmol glucose Eq./mL; EPS-b*: nmol glucose Eq./mL; EPS-b**: nmol glucose Eq./μg BSA Eq.

2.3.3 ABTS Antioxidant activity

The ABTS radical-scavenging assay has been used to assess the ability of metabolic compounds produced by the different *Lp. plantarum* strains to act as free radical scavengers or hydrogen donors. The Trolox equivalent antioxidant capacity is expressed with respect to cellular protein content. As reported in Table 2.3, antioxidant activity values are significantly different among the tested strains. In detail, the strains LP95 and LP100 exhibited the highest values, which amounted to 14.23 and 15.84 μg Trolox Eq./mg BSA Eq., respectively, while the lowest value has been found for strain LP86 (3.89 μg Trolox Eq./mg BSA Eq.).

Table 2.3 Antioxidant and β-glucosidase activities of *Lp. plantarum* strains. All values are expressed as mean ± standard deviation (n = 3).

	Lp. plantarum strains					
	LP8	**LP25**	**LP86**	**LP95**	**LP100**	**ATCC 14917**
*Antioxidant activity	6.46 ± 0.65 [c]	6.51 ± 0.38 [c]	3.89 ± 0.34 [d]	14.23 ± 1.60 [a]	15.84 ± 0.80 [a]	13.35 ± 0.26 [b]
**β-glucosidase activity	0.30 ± 0.06 [a]	0.39 ± 0.07 [a]	0.38 ± 0.08 [a]	0.45 ± 0.03 [a]	0.26 ± 0.08 [b]	0.26 ± 0.08 [b]

Different lowercase letters (a–d) in each row indicate significant differences ($p < 0.05$).
*Expressed as μg Trolox Eq./mg BSA Eq.; ** Expressed as nmol *p*-Nitrophenol/(min mL).

2.3.4 β-glucosidase activity

The enzymatic activity exhibited by the bacterial cultures was assessed as the release of *p*-NP from the *p*-NPG substrate in the time unit. As reported in Table 2.3, all tested strains showed β-

glucosidase activity, which ranged from 0.26 to 0.45 nmol *p*-NP/(min mL). The data show significant differences in this specific enzymatic activity among the strains. The minimum values were recorded for LP100 and ATCC 14917 strains, whereas the highest value corresponded to *Lp. plantarum* LP95.

2.3.5 GABA production

The biosynthetic ability of *Lp. plantarum* strains to produce GABA was assessed in LFM medium with and without L-glutamic acid (GLU). GABA contents were determined by injecting an aliquot of the cell extract supernatants (CES_{LFM}) via HPLC after precolumn OPA derivatization and the amounts were calculated as the internal peak area. Representative chromatograms are shown in Figure 2.2. The results, reported in Table 2.4 and summarized in Figure 2.3, were expressed as the ratio between the pmol of GABA and the cell proteins content (µg BSA Eq.). The GABA-specific contents in the CES_{LFM} obtained from the six *Lp. plantarum* strains examined showed significant differences as shown in Figure 2.3. Values varied within a range of 0.345 (LP25) and 2.227 pmol GABA/µg BSA Eq. (LP95).

Table 2.4 GABA production (pmol GABA/µg BSA Eq.) from the five *Lp. plantarum* strains grown in LFM medium with and without L-glutamic acid (GLU). All values are expressed as mean ± standard deviation (n = 3).

	GABA production of *Lp. plantarum* strains					
LFM medium	**LP8**	**LP25**	**LP86**	**LP95**	**LP100**	**ATCC 14917**
without GLU	0.068 ± 0.011 ^{b}	0.060 ± 0.011 ^{b}	0.047 ± 0.008 ^{b}	0.055 ± 0.008 ^{b}	0.088 ± 0.006 ^{a}	0.116 ± 0.031 ^{a}
with GLU	0.781 ± 0.055 ^{c}	0.345 ± 0.026 ^{d}	1.004 ± 0.063 ^{c}	2.227 ± 0.118 ^{a}	1.350 ± 0.111 ^{b}	1.204 ± 0.134 ^{b}

Different lowercase letters (a–d) in each row indicate significant differences ($p < 0.05$).

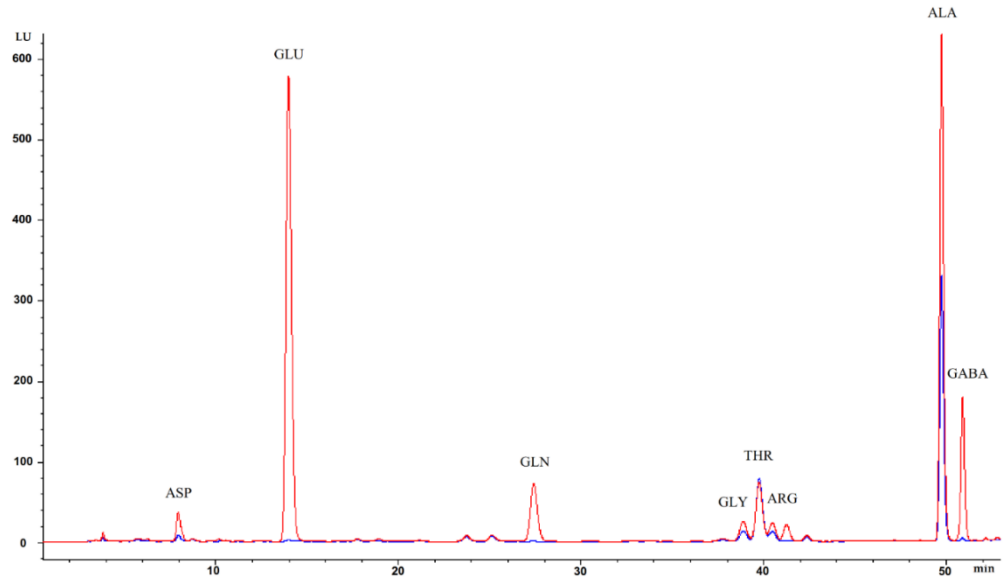

Figure 2.2 GABA production from *Lp. plantarum* LP95 in LFM medium with (■) and without (■) L-glutamic acid (GLU).

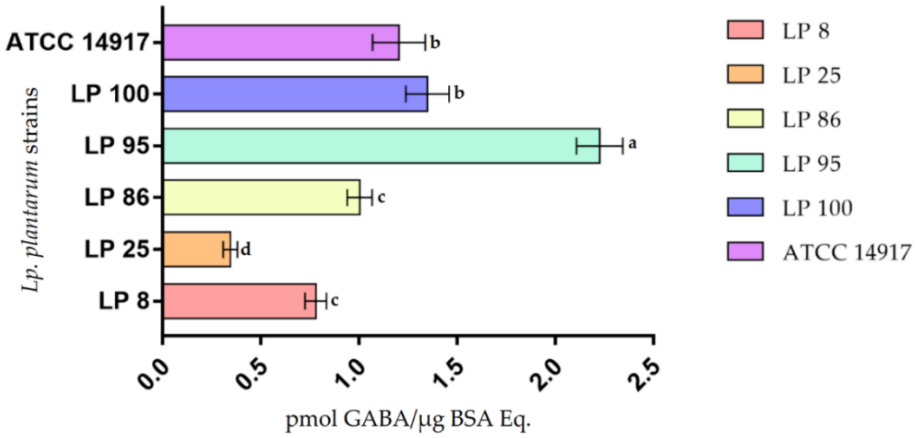

Figure 2.3 GABA amounts synthesized from *Lp. plantarum* strains grown in LFM medium with L-glutamic acid (100 µM). All data are expressed as mean ± standard deviation (n = 3). Different lowercase letters (a–d) indicate significant differences ($p < 0.05$).

2.3.6 Biogenic amine production

In the qualitative screening of biogenic amines, all tested strains (LP8, LP25, LP86, LP95, LP100) and type strain ATCC 14917 were all unable to synthesize biogenic amines, as showed by the absence of indicator changes.

2.3.7 Enzyme profile of Lp. plantarum LP95

Based on the results obtained, *Lp. plantarum* LP95 was selected for characterization of its enzyme profile. The results, as reported in Table 2.5, highlight specific enzymatic activities as the esterase, esterase lipase, leucine, valine and cystine arylamidase, acid phosphatase, naphthol phosphohydrolase, β-galactosidase, α-glucosidase β-glucosidase, and N-acetyl-β-glucosaminidase. These enzyme activities mentioned above play a pivotal role in food fermentation by impacting on the aromatic profile and the content of functional metabolites.

Table 2.5 Enzymes detected with the API ZYM system.

Enzyme	*Lp. plantarum* LP95
Control (no substrate)	-
Phosphatase alkaline	-
Esterase (C4)	+
Esterase lipase (C8)	+
Lipase (C14)	-
Leucine arylamidase	+
Valine arylamidase	+
Cystine arylamidase	+
Trypsin	-
α-Chymotrypsin	-
Acid phosphatase	+
Naphthol phosphohydrolase	+
α-Galactosidase	-
β-Galactosidase	+
β-Glucuronidase	-
α-Glucosidase	+
β-Glucosidase	+
N-Acetyl-β-glucosaminidase	+
α-Mannosidase	-
α-Fucosidase	-

The data are expressed as follows: (+) enzime expressed; (-) unexpressed enzyme.

2.4 Discussion

The characterization of microorganisms beneficial for food preservation and the prevention of pathogen growth is mainly based on their ability to produce bioactive compounds that have antimicrobial activity.

In our investigation the reference strain ATCC 14917 and the five *Lp. plantarum* isolated by us from bee bread (Iorizzo et al. 2020b, a) were able to limit the growth of indicator bacteria, but with varying degrees of intensity. The mechanisms underlying the inhibitory capacity of LABs are attributable to multiple mechanisms of action, such as individual compounds that may cause membrane destabilization (such as fatty acids or peptides), H^+ gradient interference (such as organic acids or peptides), or enzyme inhibition (such as hydroxy acids) (Siedler et al. 2019). Moreover, there may be some synergistic and/or additive effects that involve various compounds (Ibrahim et al. 2021).

Since no effect on the growth of indicator bacteria was observed using MRS pH 3.8, the inhibition of the LABs against pathogens cannot be ascribed to a simple consequence of the lowering of pH due to the bacterial metabolism of the producer strains. Antimicrobial activity, therefore, was most likely caused by the production of organic acids, protein compounds or other antimicrobial metabolites contained in CFS, which need to be further investigated.

The LAB food-grade EPSs have earned considerable attention for their structural and sensory properties in the food industry, primarily for dairy products and more recently for the improvement of cereal-based products (Galle et al. 2011; Enikeev 2012; Patel and Prajapat 2013; Chen et al. 2016; Lynch et al. 2018b, a; Silva et al. 2019; Prete et al. 2021). Furthermore, EPSs have also gained major relevance in pharmacological and nutraceutical applications, as they can serve as immunomodulatory, antioxidant, anti-cancer, cholesterol-lowering, antimicrobial, and prebiotic agents (İspirli et al. 2018; Saadat et al. 2019; Angelin and Kavitha 2020; Wu et al. 2021; Daba et al. 2021). Microbial EPSs are not permanently anchored to the microbial cell surface and exist in two forms based on their localization: cell-bound EPSs, which are tightly adhered to the bacterial surface (bound exopolysaccharides; EPS-b), and EPSs that are released into the surrounding medium (released exopolysaccharides; EPS-r). These experimental approach made it possible to quantitatively, determined the two exopolysaccharide matrices and showed the ability of the tested *Lp. plantarum* strains to produce these compounds with significant differences in EPS-b production.

These findings confirm that several *Lp. plantarum* strains are good EPS producers (Wang et al. 2015; Ayyash et al. 2018; Silva et al. 2019). In particular, the strains LP95 and LP100 produced quantities significantly higher compared to the other strains. The rheological and prebiotic properties of the EPS produced by LAB (Patel and Prajapat 2013) make it for possible technological applications in the food industry of the *Lp. plantarum* LP95 and LP100 strains.

Some LAB strains possess both enzymatic and non-enzymatic antioxidant activity, which reduces the risk of reactive oxygen species (ROS) accumulation during food ingestion, resulting in a decrease in oxidative stress (Wu et al. 2014; Tang et al. 2017; Hoffmann et al. 2019; Feng and Wang 2020). Over the last decade, considerable attention has been drawn to the use of LAB as natural antioxidants in the food industry (Kudoh et al. 2001). Apart from some cellular constituents (exopolysaccharides, peptidoglycan, lipoteichoic acid, and proteins) LABs are known to have complex antioxidant activity and different strains operate different mechanisms: chelation of toxic ions (Fe^{2+} and Cu^{2+}), synthesis of antioxidant compounds (e.g., glutathione, butyrate, folate) and activation of enzymatic pathways for the scavenging of free radicals (Liu et al. 2010; Li et al. 2012; Zhang et al. 2013; Mishra et al. 2015; Wang et al. 2017a). It has also been reported that the antioxidant activity of LABs may be linked to some proteolytic-derived peptides and may vary depending on the species and on the strain (Kudoh et al. 2001). Whereas in other studies, the antioxidant activity of various *Lp. plantarum* strains is associated with EPS (Zhang et al. 2013; Wang et al. 2017b, a).

Tang et al. (2017) reported that CFS from *Lp. plantarum* MA2 exhibited strong reducing capacity, lipid peroxidation inhibition, Fe^{2+} chelating and free radical scavenging activities. Moreover, the cell homogenate revealed glutathione peroxidase activity and superoxide dismutase activity.

In this study, antioxidant activity was evaluated by means of the ABTS assay, which is considered one of the most sensitive procedures (Chanput et al. 2016) and a valid method for the assessment of the antioxidant activity of both hydrophilic and lipophilic extracts (Arnao et al. 2001).

All five strains of *Lp. plantarum* exhibited antioxidant activity and the mechanism or molecules involved in this behavior require more detailed study.

Over recent years, the pharmaceutical and food industries' research has strongly focused on the development of dietary supplements and fermented foods enriched with GABA for the various health benefits of this amino acid, including neurostimulation and cardio protection (Diez-Gutiérrez et al. 2020; Cui et al. 2020). GABA-producing LABs may provide new opportunities for naturally fermented functional food products (Quílez and Diana 2017). LABs synthesize GABA via the GAD pathway, in which the GAD protein localized in the cytoplasm is the key enzyme. Strains with the *gad* gene are therefore able to synthesize GABA. The first step in the GAD pathway is carried out by a GLU/GABA antiporter, encoded by a *gadC* gene. This antiporter pumps the GLU precursor into the microorganism (Diez-Gutiérrez et al. 2020). LABs have strain-specific GABA synthesis capacities and several environmental factors, such as pH and temperature, impact the expression of the *gad* gene (Lin et al. 2017; Lim et al. 2018). *Lp. plantarum* is one of the most widely distributed and common bacteria occurring in fermented foods and therefore it would be valuable to achieve effective GABA production using selected strains from this species.

Our results showed that the tested *Lp. plantarum* strains are able to synthesize GABA, confirming that bacteria belonging to this species are able to produce this amino acid (Franciosi et al. 2015; Ly et al. 2019). To assess the ability to produce GABA, we used the medium LFM with a defined centesimal composition to exclude interference from undefined substances in the MRS medium based on complex matrices (e.g., yeast extract and meat extract).

LABs with the ability to synthesize GABA can offer new opportunities in the design of functional foods. However, further investigations are needed to optimize the fermentation conditions and to improve the production of this non-proteic amino acid (Tajabadi et al. 2015; Cui et al. 2020; Park et al. 2021).

Isoflavones are plant-based polyphenolic compounds, predominantly found in glycosidic forms that can be hydrolyzed by intestinal and bacterial β-glucosidases to release aglycones, which are subsequently absorbed in the large intestine (Sirilun et al. 2012; Yuksekdag et al. 2017).

β-glucosidase LAB producers have a major role in the release of free flavonoids from their glycosylate precursors found in fruits and other plant tissues, and enhance the bioavailability of these health-promoting plant metabolites (Lorn et al. 2021).

In our study, the selected *Lp. plantarum* strains exhibited β-glucosidase activity without significant differences among them. As a result of this specific enzymatic activity, it would therefore be interesting to use them as starter cultures in future applications to optimize the organoleptic, technological and nutritional properties of fermented foods (e.g., soy-derived foods or vegetable beverages) (Michlmayr and Kneifel 2014; Lorn et al. 2021).

2.5 Conclusions

This *in vitro* study allowed us to screen some *Lp. plantarum* strains possessing good potential for further studies on their probiotic and functional properties.

We found a large heterogeneity related to examined properties confirming that these are strain-specific. Therefore, our results suggest that a careful selection within a given species is of utmost importance to identify appropriate strains for specific biotechnological applications. In particular, the evaluation of our results shows that *Lp. plantarum* LP95 was the best-performing strain and that could be a candidate as new a probiotic in the para-pharmaceutical sector and as food microbial culture to improve some functional characteristics of specific products. Clearly, we consider this study a preliminary selection and further *in vitro* and *in vivo* investigations are needed for the use of this LAB in human diet. The results obtained support the possible use of *Lp. plantarum* LP95 as a fermentation starter for the production of functional soy plant-based products. Indeed, the strong activity of β-glucosidase and the ability to synthesise GABA and EPS, could lead to an increase in health-promoting compounds. Finally, the EPS could also influence the rheological properties of products.

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Chapter 3

Fermented soymilk production by *Lactiplantibacillus plantarum* LP95

3.1 Introduction

The growing interest of consumers in nutritionally enriched and health-promoting foods provokes interest in the eventual development of fermented functional foods (Shah et al. 2023). In this view, the consumption of soybean (*Glycine max*) and its products has received attention from researchers and consumers both for the nutritional value and for its potential health benefits, including reducing the risk of cardiovascular diseases, diabetes, breast cancer, and chronic inflammation, due to the presence of bioactive compounds, as the isoflavones (Messina 2016; Kumari et al. 2022). The water extract of soybeans, namely soymilk, is an important oriental traditional beverage (Van Dyck 2010), considered a colloidal system as it contains protein and minerals (Li et al. 2014). This beverage can be a good substitute for animal-based milk origin and an ideal food for vegans diets and lactose-intolerant people (Subrota et al. 2013). Despite the listed properties of soymilk, its consumption in Western countries is still limited due to its unpleasant taste and its anti-nutritional factor, as the phytates, that compromise the natural availability of minerals, as well as the presence of indigestible oligosaccharides, such as raffinose and stachyose, that cause flatulence (Zhao and Shah 2014; Theodoropoulos et al. 2018). Considering these limitations, the fermentative biotransformation approach using Lactic Acid Bacteria (LAB) is an effective strategy to improve the nutritional, textural, and rheological properties of soymilk (Kumari et al. 2022). Isoflavones are phytochemical compounds naturally present in soymilk and their enteric absorption is related to their chemical structure. The concentrations of the different forms of these bioactive compounds in soymilk are related to soybean cultivar, time of soaking, the ratio of soybean to water, filtration and thermal treatments (Chun et al. 2007; Yu et al. 2021). The principal isoflavones of soy are daidzein, genistein, and glycitein, which exist in plants conjugated with sugars (Zaheer and Humayoun Akhtar 2017; Hu et al. 2020). Glycosides can be hydrolyzed in the oral cavity (Walle et al. 2005) and, mainly in the intestine, to their aglycones, which are then absorbed in the intestine (Otieno and Shah 2007). The flavonoid glycosides are considered biologically inactive, and their bioavailability requires initial hydrolysis of the sugar moiety by intestinal β-glucosidases for uptake into the peripheral circulation (Setchell et al. 2002). Several studies have shown that it is possible to reverse the glucoside/aglycone ratio in soy food through LAB strains with β-glucosidase activity (Hur et al. 2000; Baú et al. 2015). Therefore, the use as starter of these

bacteria, with the aforementioned enzymatic activity, in the fermentation of soymilk could contribute to increasing bioavailable isoflavones (Donkor and Shah 2008). Moreover, some LABs, when assumed through the consumption of fermented foods, show antioxidant effects, which differ among species due to different concentrations and types of antioxidants and, therefore, could play a pivotal role in human health (Feng and Wang 2020; Li et al. 2021). Among LAB species, *Lactiplantibacillus plantarum* has the Qualified Presumption of Safety (QPS) status from the European Food Safety Authority (EFSA) and the Generally Recognized As Safe (GRAS) status from the US Food and Drug Administration (US FDA) (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) et al. 2018). In addition, this bacterial species has a documented history as a microbial food culture (Iorizzo et al. 2016a, b; Behera et al. 2018). Based on previous studies (Letizia et al. 2022), in this work, we used the *Lp. plantarum* LP95 strain which possesses antimicrobial, β-glucosidase activities and exopolysaccharides (EPS) production capacity, as starter to obtain fermented organic soymilk without the use of stabilizers, thickeners, and acidity regulators. Therefore, the main aim of our study was to evaluate the antioxidant capacity of fermented soymilk and its enrichment in isoflavone aglycone. Furthermore, the fermented soymilk was characterized by its flow behavior and apparent viscosity.

3.2 Materials and Methods

3.2.1 Bacterial culture

In this study, *Lp. plantarum* LP95 (Genbank accession number: OM033654), isolated from fermented pollen (bee bread), was cultured, at 37 °C, in MRS medium (de Man–Rogosa and Sharp broth; Oxoid Ltd., Hampshire, UK). After 12 hours, bacterial cells were harvested by centrifugation (10,000 rpm, 5 min, 4 °C) and washed with sterile saline solution (0.9 % *w*/*v* NaCl). The supernatant was discarded and the cell pellet was recovered, resuspended in saline solution and properly diluted to adjust cell concentration before being used as starter.

3.2.2 Chemicals

All chemicals were purchased from Sigma (Sigma-Aldrich, Merck KGaA, Darmstadt, DE). Eluents and water for analytical analysis were purified using a Milli-Q system (Millipore, Burlington, MA, USA).

3.2.3 Preparation of soymilk

Handcrafted soymilk was prepared according to Wang et al. (2006) with some modification by soaking each 400 g of mature yellow organic soybeans (*Glycine max*, marketed by BV&FdL S.r.l.;

Bentivoglio, BO, Italy) was soaked in 1200 mL sterile distilled water for 12 hours. Subsequently, soybeans were drained, dried, and blended with 1400 mL of water. The resulting product was subjected to blanching (90 °C, 10 min), afterward filtered and sterilized at 121 °C for 15 min, and finally cooled for the inoculum phase. Next, the entire mass was homogenized by stirring before adding the microbial starter. After starter inoculation, soymilk was portioned into three different sterile glass containers (200 mL) for subsequent experimental and analytical steps.

3.2.4 Screening for optimum temperature

To optimize the fermentation phase, preliminary tests were carried out inoculating the obtained soymilk with *Lp. plantarum* LP95 at a concentration of 10^6 CFU/mL at three temperatures (20 °C, 28 °C, and 37 °C) for 72 hours.

3.2.5 Production of fermented soymilk

According to the preliminary screening for the optimum temperature, the fermentation phase was conducted at 37 °C, inoculating the soymilk with *Lp. plantarum* LP95 (10^8 CFU/mL). After 24 hours, the clot was broken, and the final product was stored at 4 °C for 49 days. Samples were analyzed immediately and every 7 days until the 49th day.

3.2.6 Determination of Water Holding Capacity (WHC)

WHC is defined as the capacity of soymilk to retain all or part of its water and its determination was assayed according to the method described by Li et al. (2014). Briefly, fermented soymilk (30 g) was centrifuged at 8,000 rpm at 4 °C for 15 min. The supernatant was collected, weighed and WHC was calculated using the equation (1):

$$WHC\ (\%) = \left(1 - \frac{W_1}{W_2}\right) \times 100 \qquad (1)$$

W_1: weight of supernatant after centrifugation (g); W_2: fermented soymilk weight (g).

3.2.7 Flow behavior and apparent viscosity

The flow behavior of the fermented soymilk was evaluated by a rotational rheometer (Haake Mars III, Thermo Scientific, Karlsruhe, DE) with a plate–plate measuring system (20 mm in diameter, 1 mm gap distance). Flow curves were generated by varying the shear rate from 0.001 to 300 s^{-1} over 2 minutes, recording the shear stress and viscosity values at the temperature of 37 °C (during the fermentation step) and 4 °C (during storage). The temperature was controlled by a heating and cooling system (Phoenix II, Thermo Scientific, Karlsruhe, DE) combined with a Peltier system

(Cofelice et al. 2021, 2023). In addition, the apparent viscosity was obtained from flow curves at a shear rate of 50 s^{-1} at 4 °C (Koksoy and Kilic 2004). Unfermented soymilk was used as control.

3.3 Chemical analysis

For subsequent chemical analyses, soymilk was sampled at 0, 3, 6, 9, and 24 h from the beginning of fermentation and subjected to freeze-drying. In details, samples were frozen at − 40 °C and then freeze-dried under vacuum at 15 Pa in a freeze-drier Genesis 25 ES dryer (VirTis Genesis 25ES; SP Industries Inc., Gardiner, NY, USA) for 48 h (maximum shelves' temperature +20 °C). After freeze-drying, the samples were stored at room temperature. The freeze-drying treatment was necessary to guarantee the stability of the soymilk for the subsequent analytical steps. The moisture content (%) of the freeze-dried soymilk at was determined according to the method described by Jung et al. (2008) with a slight modifications.

3.3.1 Determination of isoflavones

The content of isoflavones in soymilk sample was determined by HPLC, using a Dionex (Sunnyvale, CA, USA) Ultimate 3000 binary pump equipped with a degasser, thermostated column compartment and a Diode Array Detector (DAD). Then an aliquot of 500 mg freeze-dried soymilk was subjected to a methanolic extraction, according to the method described by Fahmi et al. (2014). Briefly, 25 µL of methanolic extract was injected into a reversed-phase column (Phenomenex Luna 5 µm C18 100 Å 250 × 4.6 mm) protected with Security Guard Phenomenex (Torrance, CA, USA). Elution was performed at a flow rate of 1 mL/min, at 35 °C, using a solution of phosphoric acid 0.1 % (eluent A) and absolute methanol (eluent B). In detail were summarized the elution profile: %B: 0 min 28 %, 2 min 37 %, 30 min 73 %. Isoflavones were detected at a wavelength of 254 nm. The identification and quantification were performed using the Chromeleon software ver. 6.80 through retention times and calibration curves of daidzein, daidzin, genistein, genistin, glycitein, and glycitin, as references external standards.

3.3.2 Antioxidant activity (ABTS assay)

The 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) was used for estimating the Total Antioxidant Activity (TAA) in samples by decolorizations of the 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) generated in organic phase, according to the method described by Re et al. (1999). Briefly, ABTS was dissolved in methanol to a 7 mM concentration. ABTS radical cations ($ABTS^{\bullet+}$) were produced by reacting the ABTS methanol solution with 2.45 mM potassium persulfate. The $ABTS^{\bullet+}$ solution was diluted with cold pure methanol up to an optical

density (OD) of 0.700 at 745 nm. An aliquot of 20 mg of freeze-dried soymilk sample was subjected to methanolic extraction (1 mL). After 2 hours, 100 μL of methanolic extract were mixed with 900 μL of the $ABTS^{\bullet+}$ solution and the decrease of absorbance was recorded using a BioSpectrometer (Eppendorf, Hamburg, DE). Trolox was used as standard for the calibration curve and TAA were expressed as Trolox Equivalent Antioxidant Capacity (TEAC).

3.3.3 ThioBarbituric acid Reactive-Substances (TBARS) assay

The TBARS assay was used to estimate lipid peroxidation using the malondialdehyde (MDA) as a marker. To 40 mg freeze-dried soymilk, 1 mL of ethanol/water solution (80:20 *v/v*) was added, and the suspension was vigorously shaken. Then samples were centrifuged at 13,000 rpm and the supernatant was used to generate the malondialdehyde-thiobarbituric acid (MDA-TBA) complex, according to the method described by Hodges et al. (1999) with some modifications. Absorbances were detected at 532, 440 and 600 nm, using a BioSpectrometer (Eppendorf, Hamburg, DE). An aqueous solution of malondialdehyde bis (dimethyl acetal) was used as a standard for the calibration curve. The quantities of MDA during the fermentation phase were calculated using the modified equation reported by Landi (2017).

3.4 Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA), followed by Tuckey's multiple comparisons, while a *t*-test was used for the analysis of the significance of the apparent viscosity during the fermentation stage, using SPSS Statistics 21 software (IBM Corp, Armonk, NY, USA). The calculations were performed on data obtained from the analysis carried out on three independent aliquots (n = 3) obtained from a single initial mass of soymilk after starter microbial inoculation. All data were expressed as a mean ± standard deviation (± SD).

3.5 Results

3.5.1 Fermentation parameters

During fermentation phase, preliminary tests were carried out by inoculating soymilk with *Lp. plantarum* LP95 at a concentration of 10^6 CFU/mL at three different temperatures (20 °C, 28 °C, and 37 °C). The pH and viable cell counts were monitored at 0, 3, 6, 9, 24, 32, 48, and 72 hours. Based on these results, 37 °C was shown to be the optimal temperature for *Lp. plantarum* LP95 to ferment soymilk, despite the comparable trend of the fermentation kinetics. This is illustrated in Figure 3.1 and reported in Tables 3.1 and Table 3.2.

Table 3.1 Viable cell count (log CFU/mL) of *Lp. plantarum* LP95 during preliminary fermentation trial of soymilk at 20 °C, 28 °C and 37 °C. All values are expressed as mean ± standard deviation (n = 3).

Fermentation time (hours)	Viable cell count 20 °C	Viable cell count 28 °C	Viable cell count 37 °C
0	7.29 ± 0.01 [a]	7.30 ± 0.01 [a]	7.32 ± 0.02 [a]
3	7.53 ± 0.02 [c]	8.01 ± 0.03 [b]	8.19 ± 0.03 [a]
6	7.85 ± 0.01 [c]	8.70 ± 0.01 [b]	8.89 ± 0.01 [a]
9	8.25 ± 0.03 [c]	8.93 ± 0.01 [b]	9.03 ± 0.04 [a]
24	9.17 ± 0.03 [a]	9.21 ± 0.04 [a]	9.23 ± 0.05 [a]
32	9.20 ± 0.04 [a]	9.23 ± 0.04 [a]	9.25 ± 0.06 [a]
48	9.17 ± 0.05 [a]	9.20 ± 0.02 [a]	9.24 ± 0.04 [a]
72	9.16 ± 0.03 [a]	9.21 ± 0.04 [a]	9.15 ± 0.05 [a]

Different lowercase letters in each row indicate significant differences ($p < 0.05$).

Table 3.2 Changes in pH during preliminary fermentation trial of soymilk with *Lp. plantarum* LP95 at 20 °C, 28 °C and 37 °C. All values are expressed as mean ± standard deviation (n = 3).

Fermentation time (hours)	pH 20 °C	pH 28 °C	pH 37 °C
0	6.40 ± 0.02 [a]	6.40 ± 0.03 [a]	6.40 ± 0.02 [a]
3	6.30 ± 0.02 [a]	6.23 ± 0.02 [b]	6.10 ± 0.02 [c]
6	6.20 ± 0.03 [a]	5.65 ± 0.01 [b]	5.33 ± 0.01 [c]
9	5.98 ± 0.02 [a]	5.31 ± 0.02 [b]	4.94 ± 0.04 [c]
24	5.05 ± 0.03 [a]	4.73 ± 0.03 [b]	4.27 ± 0.03 [c]
32	4.95 ± 0.03 [a]	4.59 ± 0.01 [b]	4.18 ± 0.03 [c]
48	4.76 ± 0.02 [a]	4.41 ± 0.02 [b]	4.15 ± 0.02 [c]
72	4.47 ± 0.03 [a]	4.12 ± 0.02 [b]	4.10 ± 0.03 [b]

Different lowercase letters in each row indicate significant differences ($p < 0.05$).

Specifically, this was particularly evident after 9 hours of fermentation, when the viable cell counts at 37 °C gradually increased from 7.32 log CFU/mL to a value more than 9.03 log CFU/mL, while it was significantly lower at 20 °C (8.25 log CFU/mL) and 28 °C (8.93 log CFU/mL). Additionally, the fermented soymilk, as depicted in Figure 3.1, attained values below pH 5.0 after 9 hours at 37 °C. These values were significantly lower than those found in experiments at 20 °C (pH 5.98) and 28 °C (pH 5.31).

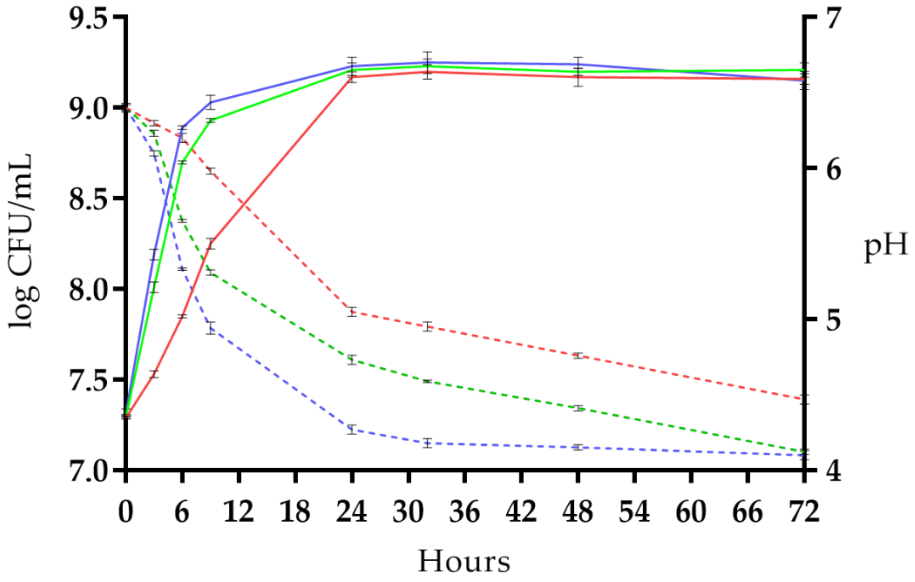

Figure 3.1 Viable cell count (log CFU/mL) of *Lp. plantarum* LP95 (continuous line) and pH values (dashed line) during fermentation of soymilk at 20 °C (red line), 28 °C (green line) and 37 °C (blue line). Error bars indicate the standard deviation of the mean (n = 3).

3.5.2 Physical-chemical and microbiological characteristics of soymilk during fermentation and storage

Soymilk samples inoculated with *Lp. plantarum* LP95 (10^8 CFU/mL) were analyzed immediately and every 7 days until the 49th day. Figure 3.2 depicts the pH values and viable cell count using *Lp. plantarum* LP95 during the soymilk's fermentation and storage stages (see also Table 3.3).

Table 3.3 Changes of viable cell count (log CFU/mL) and pH in soymilk during the fermentation (37 °C) and storage stage (4 °C) using *Lp. plantarum* LP95 as starter. All values are expressed as mean ± standard deviation (n = 3).

Stages	Time	Viable cell count	pH
Fermentation (hours)	0	8.03 ± 0.03 ^{e}	6.41 ± 0.01^{a}
	3	8.29 ± 0.02 ^{d}	5.99 ± 0.02 ^{b}
	6	9.05 ± 0.04 ^{b}	5.18 ± 0.02 ^{c}
	9	9.15 ± 0.02 ^{a}	4.82 ± 0.03 ^{d}
	24	9.20 ± 0.03 ^{a}	4.17 ± 0.03 ^{e}
Storage (days)	1	9.21 ± 0.04 ^{a}	4.16 ± 0.01 ^{e}
	7	9.05 ± 0.05 ^{b}	4.15 ± 0.01 ^{e}
	14	8.68 ± 0.01 ^{c}	4.16 ± 0.02 ^{e}
	21	8.10 ± 0.03 ^{e}	4.15 ± 0.02 ^{e}
	28	7.70 ± 0.04 ^{f}	4.15 ± 0.02 ^{e}
	35	7.60 ± 0.02 ^{g}	4.14 ± 0.03 ^{e}
	42	7.51 ± 0.01 ^{h}	4.14 ± 0.02 ^{e}
	49	7.48 ± 0.02 ^{h}	4.14 ± 0.01 ^{e}

Different lowercase letters in each column indicate significant differences ($p < 0.05$).

The viable cell count of *Lp. plantarum* LP95 reached a value of 9 log CFU/mL during the fermentation phase, which remained constant for the following seven days. The viable cell count decreased rapidly after 7 days, then more slowly from day 28 onwards. Even after 49 days of storage, the viable cell count was still above 7 log CFU/mL. The pH of the fermented soymilk showed no change during storage, whereas a considerable acidification process occurred during fermentation (from 6.5 to 4.2). Furthermore, 24 hours later, the Water Holding Capacity (WHC) of the fermented soymilk was measured, showing a value approximately 43.1 ± 0.3 %.

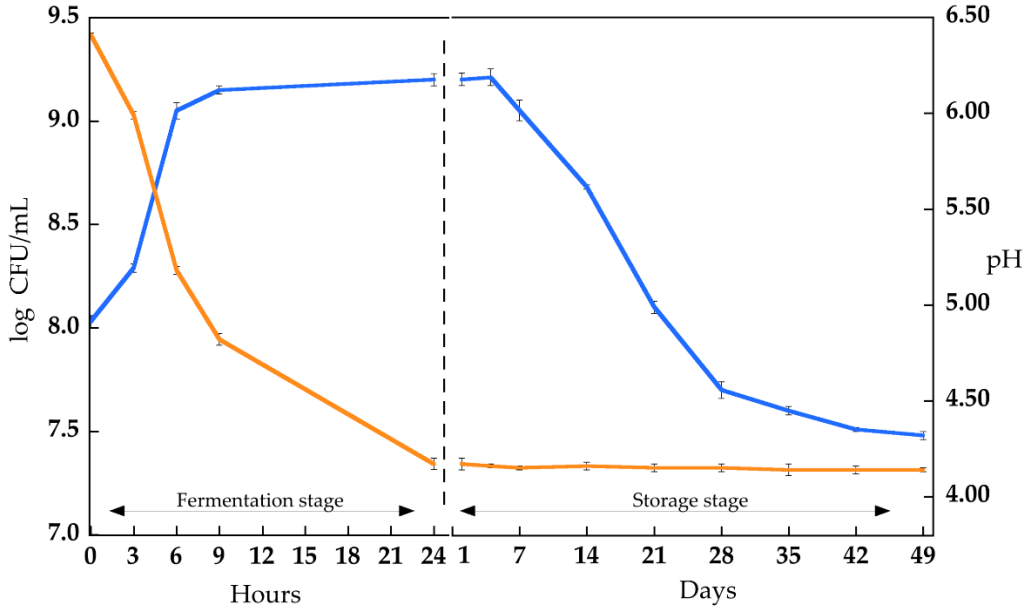

Figure 3.2 Viable cell count (log CFU/mL) of *Lp. plantarum* LP95 (blue line) and pH values (orange line) during soymilk fermentation (37 °C; 24 hours) and fermented soymilk storage (4 °C; 49 days). Error bars indicate the standard deviation of the mean (n = 3).

3.5.3 Flow behavior and apparent viscosity of fermented soymilk

It is commonly known that the flow behavior of a sample is defined by the correlation between the shear stress (τ) and the shear rate ($\dot{\gamma}$). A sample can be categorized as having non-Newtonian behavior when the viscosity (η) varies as a function of the applied shear rate or shear stress, or as having Newtonian behavior when the viscosity does not vary with deformation. The purpose of this study was to provide preliminary rheological information regarding fermented soymilk by measuring the apparent viscosity of the product. The flow behavior of fermented soymilk was assessed in both the 37 °C fermentation stage and at the 4 °C storage stage. The resulting curves are shown in Figures 3.3A and 3.3B, respectively. In detail, when the shear rate increased, the apparent viscosity dropped because all samples exhibited non-Newtonian shear-thinning behavior. Figure 3.3A shows how the soymilk viscosity changed as a result of the fermentation process, with

a noticeable increase after 24 hours. During the storage stage (Figure 3.3B), a slight decrease in the viscosity of the sample was observed, starting after 21 days. The consistency coefficient (*K*) and flow behavior index (*n*) were obtained as described by Hamet et al. (2015) fitting the flow curves with the Ostwald de Waele model and results are reported in Table 3.4. Additionally, the apparent viscosities of fermented soymilk at a shear rate of 50 s^{-1} was assessed during the fermentation and storage stages, as depicted in Figures 3.4A and 3.4B and reported in Table 3.5 and Table 3.6, respectively. Specifically, significant differences in apparent viscosity were detected within the sample at 0 hours and after 24 hours of fermentation. However, after clot rupture at 7 days of storage (4 °C), the highest level of apparent viscosity occurred, while at 21 days, a rapid decline was observed.

Table 3.4 Values of consistency coefficient (*K*) and flow behavior index (*n*) obtained by fitting the flow curves with Ostwald de Waele model. All values are expressed as mean ± standard deviation (n = 3).

	K	***n***	$\mathbf{R^2}$
Fermentation stage (37 °C)			
0 hour	0.70 ± 0.04 ^{b}	0.68 ± 0.01 ^{a}	0.992
24 hours	5.27 ± 0.23 ^{a}	0.41 ± 0.01 ^{b}	0.990
Storage stage (4 °C)			
0 day	7.40 ± 0.19 ^{a}	0.39 ± 0.01 ^{b}	0.995
21 days	7.18 ± 0.25 ^{a}	0.35 ± 0.01 ^{c}	0.986
49 days	3.36 ± 0.21 ^{b}	0.44 ± 0.01 ^{a}	0.977

Different lowercase letters in each column indicate significant differences ($p < 0.05$).
Ostwald de Waele model: $\tau = K*\dot{\gamma}^n$
where τ is the shear stress (Pa), $\dot{\gamma}$ the shear rate (1/s), *K* is the consistency coefficient ($Pa*s^n$), corresponding to the fluid consistency and *n* is the flow behaviour index (dimensionless number).
For *n* values = 1 the fluid behaves as a Newtonian fluid, for $n < 1$ the fluid is considered pseudoplastic or shear thinning fluid, and for $n > 1$ the fluid behaves as dilatant or shear thickening fluid.

Table 3.5 Apparent viscosity at 50 s^{-1} of soymilk during fermentation stage at 37 °C using *Lp. plantarum* LP95 as starter. All values are expressed as mean ± standard deviation (n = 3).

	Fermentation stage (hours)	
Apparent viscosity	0	24
(Pa s)	0.195 ± 0.003 ^{a}	0.573 ± 0.028 ^{b}

Different lowercase letters in row indicate significant differences ($p < 0.05$).

Table 3.6 Apparent viscosity at 50 s^{-1} of fermented soymilk during storage stage at 4 °C using *Lp. plantarum* LP95 as starter. All values are expressed as mean ± standard deviation (n = 3).

Storage stage 4 °C (days)	Apparent viscosity (Pa s)
0	0.670 ± 0.018 [a]
7	0.706 ± 0.045 [a]
14	0.628 ± 0.068 [a]
21	0.564 ± 0.033 [b]
28	0.492 ± 0.030 [b]
35	0.434 ± 0.027 [c]
42	0.397 ± 0.022 [c]
49	0.368 ± 0.021 [c]

Different lowercase letters (a–c) in each row indicate significant differences ($p < 0.05$).

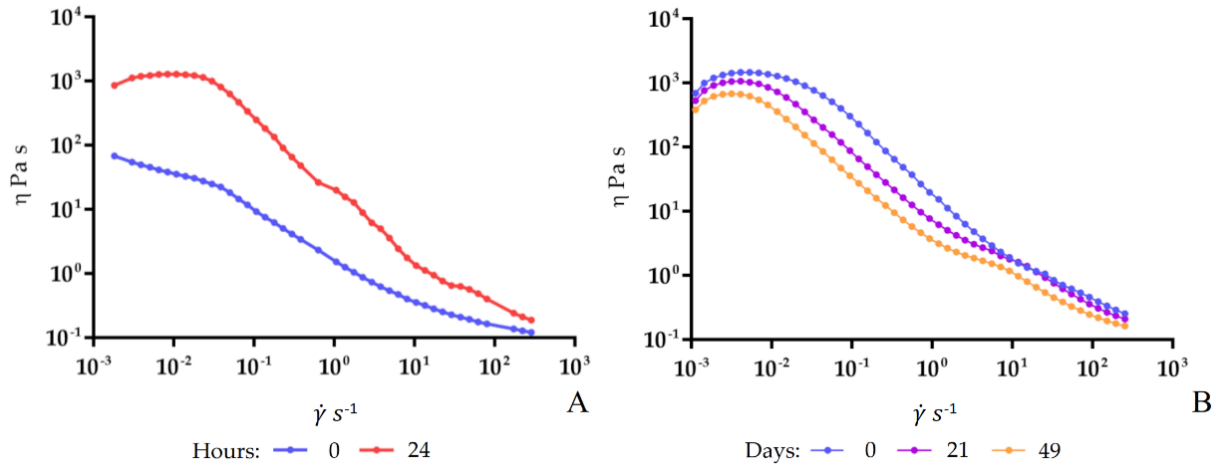

Figure 3.3 Viscosity curves of soymilk during fermentation using *Lp. plantarum* LP95 as starter at 37 °C (A), and fermented soymilk during storage at 4 °C (B) at different times: viscosity (η) as function of the shear rate ($\dot{\gamma}$).

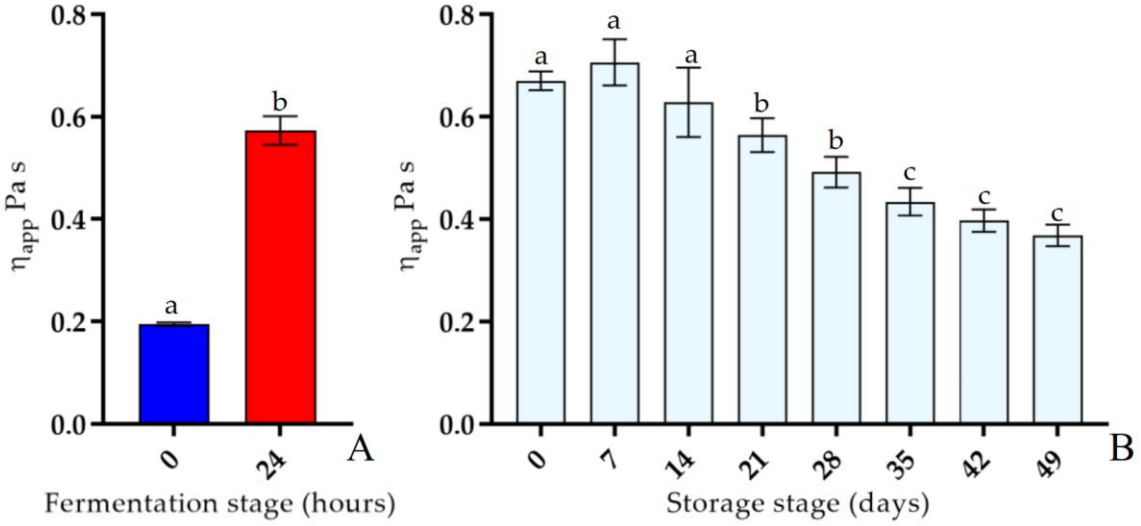

Figure 3.4 Apparent viscosity at 50 s^{-1} of soymilk during fermentation using *Lp. plantarum* LP95 as starter at 37 °C (A), and fermented soymilk during storage at 4 °C (B) at different times. All values are expressed as mean ± standard deviation (n = 3). Different lowercase letters in each bar indicate significant differences ($p < 0.05$).

3.5.4 Bioconversion of isoflavones during fermentation

Isoflavone levels in aglycone form resulted considerably increased by soymilk fermentation employing *Lp. plantarum* LP95 as a starter. The results, listed in Table 3.7 and expressed as μmol/100 g dry weight (D.W.), showed that the glycosylated isoflavones in soymilk contained daidzin, glycitin, and genistin (11.26 ± 0.27, 39.34 ± 1.00, 40.39 ± 1.27 μmol/100 g D.W.), immediately after the inoculum (0 hours), whereas their respective aglycones derivatives daidzein, glycitein, and genistein were present at a concentration of 25.64 ± 0.02, 1.58 ± 0.18 and 21.58 ± 0.39 μmol/100 g D.W., respectively. After a 24 hours fermentation, glycosylated isoflavones were no more present in the fermented soymilk, whereas the amounts of daidzein, glycitein, and genistein grew and reached their maximum values of 48.45 ± 1.30, 5.10 ± 0.16, and 56.35 ± 1.02 μmol/100 g D.W., respectively. The moisture content (%) of freeze-dried soymilk was also measured and had a value of 0.912 ± 0.005.

Table 3.7 Deglycosylation of soymilk isoflavones into aglycones during the fermentation stage of soymilk using *Lp. plantarum* LP95 as starter. All values are expressed as mean ± standard deviation (n = 3).

Isoflavones	**Fermentation stage (hours)**				
(μmol/100 g D.W.)	**0**	**3**	**6**	**9**	**24**
Daidzin	11.26 ± 0.27 ^{a}	11.08 ± 0.26 ^{a}	0.57 ± 0.02 ^{b}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}
Glycitin	39.34 ± 1.00 ^{a}	37.78 ± 0.55 ^{b}	1.45 ± 0.10 ^{c}	0.00 ± 0.00 ^{d}	0.00 ± 0.00 ^{d}
Genistin	40.39 ± 1.27 ^{a}	40.11 ± 0.02 ^{a}	4.60 ± 0.09 ^{b}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}
Daidzein	25.64 ± 0.02 ^{b}	25.76 ± 1.11 ^{b}	47.71 ± 0.72 ^{a}	48.45 ±1.74 ^{a}	48.45 ± 1.30 ^{a}
Glycitein	1.58 ± 0.18 ^{d}	1.65 ± 0.04 ^{d}	2.61 ± 0.17 ^{c}	3.69 ± 0.01^{b}	5.10 ± 0.16 ^{a}
Genistein	21.58 ± 0.39 ^{c}	21.77 ± 0.80 ^{c}	51.14 ± 0.84 ^{b}	54.75 ± 2.11^{a}	56.35 ± 1.02 ^{a}

Different lowercase letters (a–d) in each row indicate significant differences ($p < 0.05$).

3.5.5 Antioxidant activity variations during fermentation

The analysis of TAA levels during the fermentation stage, revealed that they differed significantly from soymilk immediately after the inoculum (0 hours). Specifically, the TEAC expressed as mg Trolox Eq./100 g of D.W. at 0, 3, 6, 9, 24 hours was 123.35 ± 1.58, 110.30 ± 6.03, 121.42 ± 5.26, 105.79 ± 2.34 and 108.76 ± 3.25 mg Trolox Eq./100 g D.W., as reported in Figure 3.5A and Table 3.8. In detail, the percentage decrease of TEAC in comparison to the sample at the start of fermentation was approximately 10 % after 3 hours, just 2 % after 6 hours and almost 13 % after 9 and 24 hours of fermentation.

Table 3.8 Variations of Trolox Equivalent Antioxidant Capacity (TEAC) in soymilk during fermentation stage using *Lp. plantarum* LP95 as starter. All values are expressed as mean ± standard deviation (n = 3).

	Fermentation stage (hours)				
ABTS antioxidant assay	0	3	6	9	24
(mg Trolox Eq./100 g D.W.)	123.35 ± 1.58 ^{a}	110.30 ± 6.03 ^{b}	121.42 ± 5.26 ^{a}	105.79 ± 2.34 ^{b}	108.76 ± 3.25 ^{b}

Different lowercase letters (a–b) in row indicate significant differences ($p < 0.05$).

3.5.6 Variation of MDA during fermentation

Lipid peroxidation was also assayed during soymilk fermentation, by applying the TBARS assay using the malondialdehyde (MDA) as marker (Figure 3.5B). Specifically, changes in MDA levels were measured and expressed as µg MDA Eq./100 g D.W. at 0, 3, 6, 9, 24 hours of fermentation, revealing significant variations during the progression of fermentation phase. As indicated by Figure 3.5B and Table 3.9, the maximum MDA concentration was found at 0 hours (97.11 ± 1.33 µg MDA Eq./100 g D.W.); nevertheless, it significantly decreased at 3 hours (80.26 ± 2.12 µg MDA Eq./100 g D.W.); no significant differences were found between the detected values between 6 and 9 hours (65.53 ± 1.30; 66.68 ± 1.35 µg MDA Eq./100 g D.W., respectively). At last, the MDA levels (40.17 ± 1.88 µg MDA Eq./100 g D.W.) dropped to its lowest levels after 24 hours.

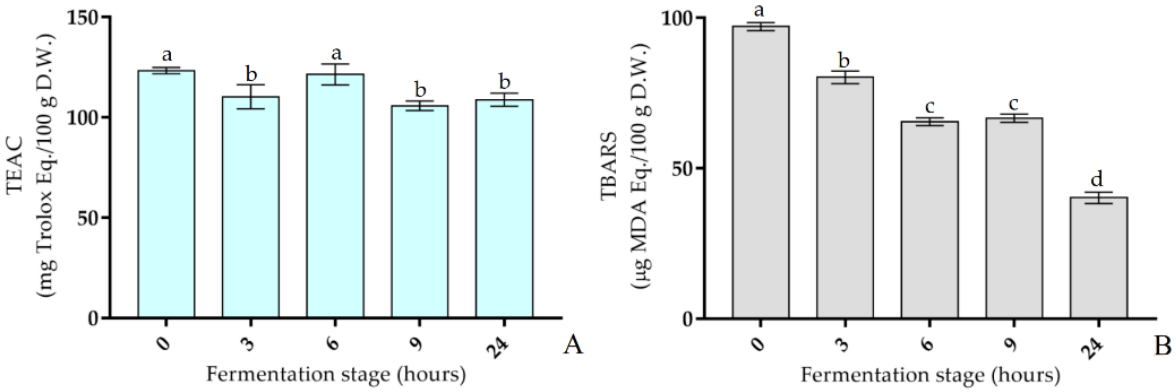

Figure 3.5 Variations in TEAC expressed as mg Trolox Eq./100 g D.W. (A) and TBARS expressed as µg MDA Eq./100 g D.W. (B) in soymilk during fermentation using *Lp. plantarum* LP95 as starter. All values are expressed as mean ± standard deviation (n = 3). Different lowercase letters in each bar indicate significant differences ($p < 0.05$).

Table 3.9 Variations of Thiobarbituric Acid Reactive-Substances (TBARS) in soymilk during fermentation stage using *Lp. plantarum* LP95 as starter. All values are expressed as mean ± standard deviation (n = 3).

	Fermentation stage (hours)				
TBARS	0	3	6	9	24
(µg MDA Eq./100 g D.W.)	97.11 ± 1.33 [a]	80.26 ± 2.12 [b]	65.53 ± 1.30 [c]	66.68 ± 1.35 [c]	40.17 ± 1.88 [d]

Different lowercase letters (a–d) in row indicate significant differences ($p < 0.05$).

3.6 Discussion

A significant area of study in the field of food biotechnology is the development of functional foods through the use of fermentative starters with probiotic properties. In this study, *Lp. plantarum* LP95 demonstrated good fermentation performances along with some attributes that enhance the overall bio-functional qualities of unfermented soymilk. Importantly, it has been suggested that the naturally occurring carbohydrates found in soymilk, such as stachyose, raffinose, sucrose, glucose and fructose, serve as substrates for LAB fermentation, which lowers

pH since lactic acid is released (Garro et al. 2004; Brereton and Green 2012). The use of *Lp. plantarum* LP95 as a started, lead to the production of a compact clot after 24 h of fermentation at 37 °C. WHC value after the clot rupture was consistent with data from other authors using various LAB strains in soybean-derived products (Mei et al. 2017; Huang et al. 2022; Baygut et al. 2023). Protein coagulation process might also be responsible for the viscosity changes that were observed during fermentation (O′ Flynn et al. 2021). In fact, as Xu et al. (2019) have reported, *Lp. plantarum*'s protease activity may actually partially hydrolyze soy proteins by reducing the pH of soymilk. Consequently, the denatured soy proteins are negatively charged, which causes increasing coagulation. Additionally, the coagulation may also be promoted by the production of EPSs from *Lp. plantarum* LP95 (Letizia et al. 2022), that are known to have a positive influence on the coagulation phase (Li et al. 2014). In order to shed light on this particular aspect, future research will compare soymilk fermented by *Lp. plantarum* LP95 with and without the addition of EPS (Zannini et al. 2016), utilizing as reference non-EPS producing LAB strains or coagulation with calcium chloride (Shun-Tang et al. 1999; Liu and Chang 2004).

The analysis of apparent viscosity is crucial to determine how fermentation may affect the finished product's sensory quality. In fact, a variety of parameters, including the kind of microorganisms employed, the duration and the temperature at which fermentation occurs, widely affect the characteristics of fermented products (Peng et al. 2022). The samples investigated in this study exhibited a non-Newtonian shear-thinning behavior, meaning that as the shear rate increased, the apparent viscosity dropped. Of note, other varieties of fermented vegetable milk also exhibited this characteristic (Yovanoudi et al. 2013; Hamet et al. 2015; Mei et al. 2017). The fermented product's flow behavior was then monitored throughout the refrigerated storage phase. Up until the fourteenth day of storage, the flow behavior was the same as the behavior during fermentation phase, then viscosity values decreased in the days that followed. Moreover apparent viscosity at a shear rate of 50 s^{-1} was determined, since physicochemical changes could also have an impact on the final product's texture and mouthfeel, that influence oral perception during the consumption of semisolid products (Arancibia et al. 2015; Pang et al. 2019). Of note, as reported in several studies, the ability of this *Lactobacillus* to increase the viscosity of soymilk could also be related to the probable presence of EPSs, (Hamet et al. 2015; Masiá et al. 2021; Jurášková et al. 2022; Zhao and Liang 2022). In this regard, we can say that the apparent viscosity of samples containing fermented soymilk kept at 4 °C throughout storage stayed broadly constant.

Enzymatic hydrolysis of the sugar moiety is instrumental to the bioavailability of glycosidic isoflavones. This process takes place in the microvillous zone of intestinal enterocytes (Hendrich 2002). Specific β-glucosidase enzyme activity is present in some LABs, which hydrolyzes

isoflavone glycosides to aglycones (Rekha and Vijayalakshmi 2011). In particular, the full biotransformation of isoflavone glucosides into their aglyconic forms was demonstrated by soymilk fermentation employing *Lp. plantarum* LP95 as starter culture, which occurred after 6 hours of fermentation. Vegetable polyphenols consumed in the diet with soymilk work as an exogenous antioxidant system. In fact, they show a significant antioxidant activity, that counteract the oxidative stress and cell damage caused by free radicals (de Mello Andrade and Fasolo 2014). The ABTS radical scavenging method was used to determine the total antioxidant level of fermented soymilk samples. In the first 3 hours of the fermentation process, the antioxidant activity slightly decreased, probably due to a depletion of antioxidant molecules by bacterial cells used to mitigate oxidative stress (Feng and Wang 2020). After six hours, the fermented soymilk's antioxidant activity increased, most likely as a result of isoflavone glycosides being bioconverted by bacteria and deglycosylated. Subsequently, the antioxidant activity declines again, probably due to the scavenging of isoflavonoids (Cetinkaya et al. 2017) produced during bacterial metabolism. The oxidative state of the final product is another crucial parameter to keep in consideration in food processing. The well-studied process of lipid oxidation proceeds via a free-radical chain mechanism, producing numerous by-products. The most prominent of these is MDA, which in biological systems is actually a marker of oxidative damage (Fernández et al. 1997). It is crucial to monitor on lipid oxidation because it causes food to lose essential nutrients and develop harmful substances that may lead to several diseases, including atherosclerosis (Spickett et al. 2015).

A method that is commonly used to assess lipid oxidation is the detection of TBARS. Nevertheless, this analysis does not reveal information about the antioxidant mechanism of action (Ghani et al. 2017). Notwithstanding these possible limitations, the TBARS test is still an advantageous complementary approach to other popular screening tests like the TAA. In our investigation, the TBARS assay demonstrated a progressive reduction in MDA levels during the fermentation of soymilk by *Lp. plantarum* LP95.

3.7 Conclusions

The results obtained in this first experimental phase have shown that the *Lp. plantarum* LP95 strain has a good potential to improve the rheological and nutritional properties of soymilk.

The enhancement of the antioxidant activity of soymilk has been shown. The increase of isoflavone aglycone contents during fermentation was a result of β-glucosidase activity toward isoflavone glucosides. This process allowed obtaining a soy beverage with enhanced antioxidant capacity able to contribute to the health and nutritional status improvement of consumers. Furthermore, we

also report that although not using chemical additives, the soymilk obtained and stored at 4 °C has a good shelf life of up to 14 days. Based on this evidence, *Lp. plantarum* LP95 appears to be a promising candidate as starter culture to obtain a fermented soymilk, rich in isoflavone aglycones and with enhanced health-promoting properties. To validate this application, future investigations may help to deepen our knowledge about the shelf life and the organoleptic and nutritional qualities of soymilk fermented by this bacterium.

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Chapter 4

Fermented tofu production by *Lactiplantibacillus plantarum* LP95

4.1 Introduction

Tofu is a traditional soybeans-derived product, which has gained great commercial interest, because of its inclusion in vegetarian, vegan, and hypocaloric diets. It has a soft and gel-like consistence, and is obtained from heat-treated soymilk, subsequently chemically coagulated, and then molded and pressed. Because of its high nutritional value and multiple potential health benefits, tofu is popular worldwide. The quality of tofu may be affected by multiple factors, but most of all the coagulation of soymilk particles induced by acidification or salting is the most important step in the tofu-making process. In fact, production technology greatly influences the yield, nutritional value and organoleptic quality of tofu products (Zhang et al. 2018b).

4.2 Materials and Methods

4.2.1 Production of tofu

The soymilk obtained from 1600 g of yellow soybeans was prepared as previously described in section 3.2.3 subsequently, was shaken, divided into 3 sterile glass containers and inoculated with *Lp. plantarum* LP95 in the same condition as described in section 3.2.5. After 24 h at 37 °C nigari 3 % *w*/*v* (marketed by La finestra sul cielo, by Natura S.r.l., Villareggia, IT) was added to the fermented product to promote the chemical coagulation. After 1 hour, the fermented soymilk was poured into disposable plastic molds for the draining of the whey, allowed to stand for 1 hour and finally was stored in a sealed sterile bag at 4 °C for 24 hours. Finally, the sample was transferred and stored for 21 days at 4 °C in sterile containers. A flowchart for fermented tofu making is shown in Figure 4.1.

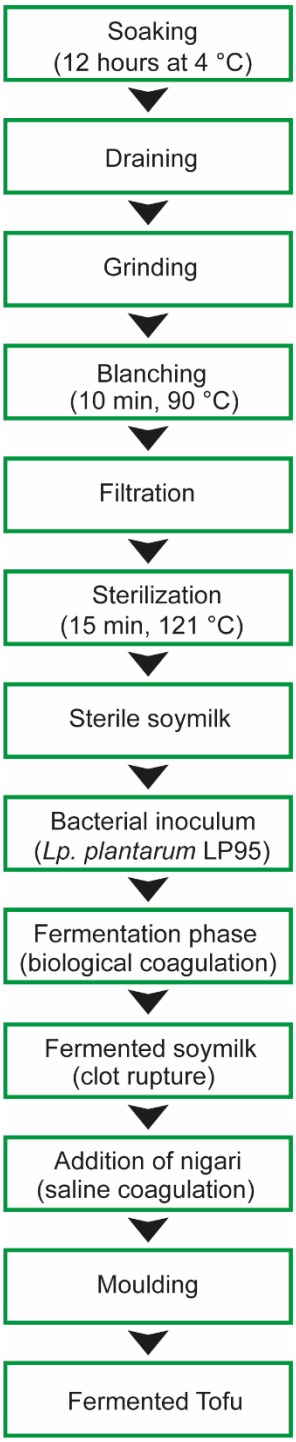

Figure 4.1 Flow chart of fermented tofu production.

4.2.2 Physical parameters

Spontaneous syneresis from tofu was performed according to the method described by Amatayakul et al. (2006) with some modifications. Briefly, the fermented tofu samples were poured into plastic molds and, after 1 hour, were stored at 4 °C for 24 hours into sealed sterile bags. The total liquid exuded during this 24 h time period was measured and the spontaneous syneresis of fermented tofu was calculated by equation (2) (Cui et al. 2021). The moisture content (%) of the freeze-dried

soymilk was determined according to the method described by Jung et al. (2008) with slight modifications, while water activity (a_w) of freeze-dried samples was measured by using water activity meter (AquaLab CX-2; Pullman, WA, USA) at 25 ± 1 °C (Singh et al. 2022).

$$Syneresis\ (\%) = \frac{W_i - W_f}{W_i - W_c} \times 100 \qquad (2)$$

Where W_i is the initial weight (g) of the plastic molds with tofu and W_f is the final weight (g) of plastic molds with tofu after whey removal and W_c is the weight (g) of the plastic molds.

4.3 Chemical analysis

To perform the chemical evaluations, unfermented soymilk, fermented soymilk (after 24 hours of fermentation) and tofu samples (after 21 day of maturation) were frozen at − 40 °C and then freeze-dried under vacuum at 15 Pa in a freeze-drier Genesis 25 ES dryer (VirTis Genesis 25ES; SP Industries Inc., Gardiner, NY, USA) for 48 h (maximum shelves' temperature +20 °C). After freeze-drying, the samples were stored at room temperature. Isoflavones content and antioxidant activity (ABTS), were evaluated using the methods described in sections 3.3.1 and 3.3.2, respectively.

4.3.1 Sugars metabolism

The sugars content was determined on an aliquot of 500 mg of fresh soymilk at 0, 3, 6, 9, 12, 18, and 24 hours of fermentation. The samples were properly diluted (1:10 - 1:40) in ultrapure water, centrifugated at 14,000 rpm for 10 min at 4 °C and supernatants were filtered through a 0.44 μm nylon syringe filter (Merck Millipore, Burlington, MA, USA) and stored at 4 °C for subsequently analytical steps. Sugars separation and detection were performed via High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using the Dionex system ICS6000 (Thermo Fisher Scientific Waltham, MA, USA). Briefly, 20 μL of sample were injected into a Dionex Carbopac PA100 analytical column (250 × 2 mm) (Thermo Fisher Scientific Waltham, MA, USA) and the elution was performed at a flow rate of 0.25 mL/min at 25 °C using eluent A (water) and eluent B (NaOH 100 mM), setting the following gradient elution profile: %B: 0 min 17 %, 8 min 17 %, 35 min 100 %, 45 min 100 %, 45.1 min 83 %, 55.0 min 83 %. The waveform time/potential expressed as second/Volt were: 0.00/0.10, 0.20/0.10, 0.40/0.10, 0.41/-2.00, 0.42/-2.00, 0.43/0.60, 0.44/-0.10, 0.50/-0.10. The flow-through electrochemical cell consisted of a 1 mm diameter gold working electrode, and an Ag/AgCl reference electrode. The instrument control, data collection and total quantification of sugars was were managed using a Chromeleon 7 (Thermo Fisher Scientific Waltham, MA, USA), comparing the retention times and internal peak areas of reference standards.

4.3.2 Amino acid (AA) profile

The primary L-amino acids content of freeze-dried samples (20 mg) was determined after ethanol/water (40/60 *v*/*v;* 1 mL) extraction. Subsequently, samples were centrifugated at 14,000 rpm for 5 min at 4 °C and supernatants were filtered through a 0.44 μm nylon syringe filter (Merck Millipore, Burlington, MA, USA) and used for amino acid derivatizations and quantification using a Prominence/Nexera X2 HPLC system (Shimadzu, Kyoto, JP), equipped with Degasser DGU-20A5R, Binary Pump LC-30AD, Autosampler SIL-30AC, Column Oven CTO-20A, DAD Detector SPD-M20A and Fluorescence Detector RF-20Axs. Briefly, about the automatic pre-column derivatization, 20 μL of extract were mixed to 40 μL of *o*-Phthalaldehyde (OPA) reagent (Agilent, Santa Clara, CA, USA). Finally, 30 μL of OPA-derivatives were injected into a reverse-phase column (ZORBAX Eclipse Plus C18, 4.6 × 250 mm 5 μm; Agilent, Santa Clara, CA, USA), protected with a SecurityGuard Phenomenex (Torrance, CA, USA). The elution was performed at a flow rate of 0.85 mL/min, at 27 °C, using eluent A (sodium acetate 50 mM / tetrahydrofuran 0.3 % / methanol 20 %, pH 6.4) and eluent B (methanol 100 %) (Carillo et al. 2019). The fluorescence of OPA derivatives was detected at 340 nm excitation and 450 nm emission wavelengths. The identification and quantification of amino acids was carried out using a LabSolutions LCGC v. 5.90 software (Shimadzu, Kyoto, JP), comparing the retention times and internal peak areas of reference standards. The content of the secondary amino acid proline was conducted in the same hydroalcoholic extracts using a ninhydrin-based method (Carillo et al. 2020).

4.3.3 Antioxidant activity (DPPH assay)

DPPH radical scavenging is a popular spectrophotometric method that has a wide used for determining the total antioxidant activity. The DPPH assay measures the TAA decolorizations of the 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical generated in organic phase, using method described by Rahman et al. (2015) with some modifications. Briefly, an aliquot of 40 mg of freeze-dried samples were subjected to methanolic extraction (1 mL), after 2 hours 100 μL of methanolic extract were mixed with 900 μL of the 70 μM DPPH$^{\bullet}$ methanol solution and the decrease of absorbance at 515 nm after 15 minutes was recorded, using a BioSpectrometer (Eppendorf, Hamburg, DE). Trolox was used as standard for the calibration curve and TAA were expressed as Trolox Equivalent Antioxidant Capacity (TEAC).

4.3.4 Total phenolics

The total phenolic content (TPC) was determined using the Folin–Ciocalteu method (Magalhães et al. 2010). In details, an aliquot of 40 mg of freeze-dried samples were subjected to methanolic

extraction (1 mL) and stored for 2 hours at 4 °C. Finally, 50 µL of extract were mixed with 50 µL of the Folin–Ciocalteu reagent (1:5 *v*/*v*) and then with 100 µL of 350 mM sodium hydroxide solution. Changes of absorbance was recorded using a BioSpectrometer (Eppendorf, Hamburg, DE) setting the wavelength at 760 nm. Gallic acid monohydrate (GA) was used as standard for the calibration curve.

4.4 Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA), followed by Tuckey's multiple comparisons using SPSS Statistics 21 software (IBM Corp., Armonk, NY, USA). Data analysis was performed from three independent aliquots (n = 3) and all data were expressed as a mean ± standard deviation (± SD).

4.5 Results

4.5.1 Fermentation kinetics

The viability of *Lp. plantarum* LP95 was monitored during fermentation and tofu production until the 21th day of maturation (Table 4.1). In details, after 24 hours of fermentation the viable cell count increased from 8.0 to 9.5 log CFU/mL approximately, while during the storage phase of tofu at 4 °C after 7 days, a fast decrease in viable cell count occurred, until reaching 7.7 log CFU/g, while at 14th and 21st days declines to about 6.0 e 5.8 log CFU/g, respectively.

Table 4.1 Viable cell count of *Lp. plantarum* LP95 during fermentation of soymilk (log CFU/mL) at 37 °C and of tofu storage (log CFU/g) at 4 °C. All values are expressed as mean ± standard deviation (n = 3).

	Days of soymilk fermentation		**Days of Tofu storage**		
	0	1	7	14	21
Viable cell count	8.07 ± 0.06 ^{b}	9.52 ± 0.11 ^{a}	7.76 ± 0.29 ^{b}	6.00 ± 0.50 ^{c}	5.83 ± 0.23 ^{c}

Different lowercase letters (a–c) in row indicate significant differences ($p < 0.05$).

Regarding the pH value, as reported in Table 4.2 and depicted in Figure 4.2, an acidification process was evident during the fermentation phase from pH 6.4 to pH 4.1, reaching a final value, after 21 days, of 3.82 in tofu.

4.5.2 Physical parameters

The syneresis value of fermented tofu after 24 hours reached a value of 22.0 ± 2.6 %. The moisture content in the freeze-dried unfermented, fermented soymilk, and tofu samples were 3.30 ± 0.10

%, 3.73 ± 0.49 % and 4.1 ± 0.93 % respectively, while a_w in the same sample were 0.26 ± 0.05, 0.21 ± 0.02 and 0.16 ± 0.01 respectively.

Table 4.2 Changes in pH of soymilk during the fermentation by *Lp. plantarum* LP95. All values are expressed as mean ± standard deviation (n = 3).

Fermentation time (hours)	pH
0	6.40 ± 0.01 [a]
1	6.20 ± 0.04 [b]
2	5.97 ± 0.05 [c]
3	5.62 ± 0.03 [d]
4	5.27 ± 0.04 [e]
5	5.02 ± 0.07 [f]
6	4.86 ± 0.03 [g]
7	4.77 ± 0.05 [g]
8	4.71 ± 0.05 [h]
16	4.13 ± 0.02 [i]
24	4.10 ± 0.01 [i]

Different lowercase letters (a–i) in each row indicate significant differences ($p < 0.05$).

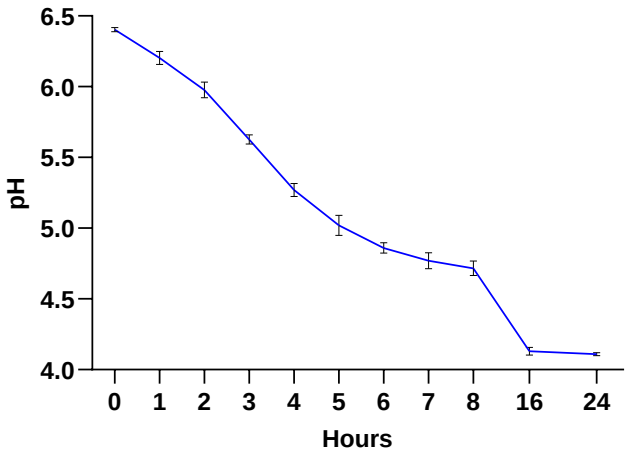

Figure 4.2 Acidification kinetic of soymilk during fermentation phase (24 hours) using *Lp. plantarum* LP95 as starter. Error bars indicate the standard deviation of the mean (n = 3).

4.5.3 Quantitative changes in sugars during fermentation of soymilk

The ability of LAB to fermenting soymilk sugars is related to a specific enzymatic activity. In particular, the chromatogram below (Figure 4.3) showed the separation of the soymilk monosaccharides (D-glucose, D-fructose and D-galactose), disaccharide (D-sucrose) and oligosaccharides (D-raffinose and D-stachyose), detected at 0, 3, 6, 9, 12, 18, and 24 hours of fermentation after *Lp. plantarum* LP95 inoculum. In detail, the fermentation curves of soymilk carbohydrate were showed in Figure 4.4 and numerical data were reported in Table 4.3. These

results highlight that carbohydrate glucose, fructose, galactose, and sucrose were catabolized while the content of raffinose and stachyose remain unchanged during 24 hours of fermentation. Specifically, after 3 hours of fermentation, the glucose, fructose, and sucrose contents decreased by 65.6, 42.7 and 11.1 % respectively, while the galactose content remained unchanged. However, after 6 hours of fermentation, a rapid decline of galactose level 86.0 % was detected, while glucose, fructose, and sucrose content decreased of 14.0, 19.1 and 19.4 %. After 9 hours of fermentation a slowing of sugar catabolism was detected, lasting up to 12 hours, when more than 90 % of monosaccharides result fermented, while about 50 % of sucrose remained unfermented. At 18 hours fermentation monosaccharides resulted completely fermented, while sucrose resulting fermented at 87.0 %. Finally, after 24 hours the fermented sucrose was at 92.7 %, leaving 7.3 % not fully fermented.

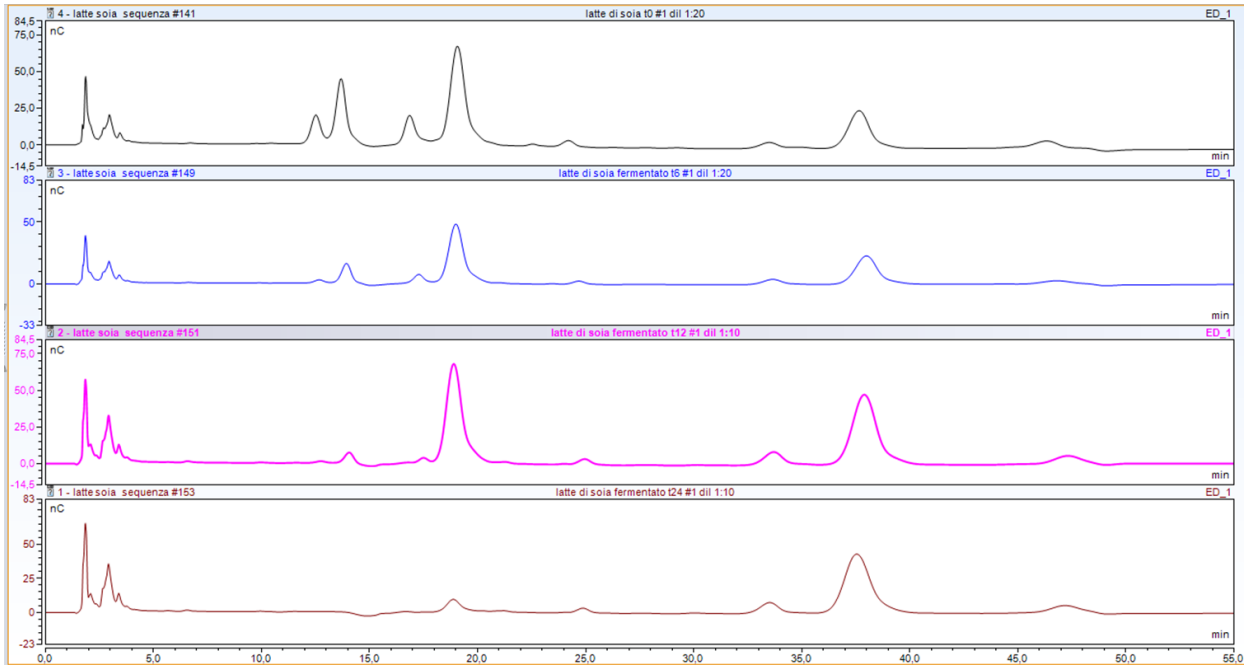

Figure 4.3 Representative chromatograms of sugars detected by HPLC in unfermented soymilk (black line) and fermented soymilk at 6 (blue line), 12 (pink line) and 24 (red line) hours of fermentation using *Lp. plantarum* LP95 as starter.

Table 4.3 Variations in sugar content of soymilk during the fermentation by *Lp. plantarum* LP95. All values are expressed as mean ± standard deviation (n = 3).

Sugars	Fermentation time (hours)						
(mg/g F.W.)	0	3	6	9	12	18	24
D-Galactose	0.43 ± 0.04 ^{a}	0.43 ± 0.03 ^{a}	0.06 ± 0.01 ^{b}	0.02 ± 0.01 ^{b}	0.02 ± 0.01 ^{b}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}
D-Glucose	0.93 ± 0.14 ^{a}	0.32 ± 0.06 ^{b}	0.19 ± 0.02 ^{b}	0.17 ± 0.02 ^{b}	0.06 ± 0.01 ^{c}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}
D-Fructose	0.89 ± 0.20 ^{a}	0.51 ± 0.03 ^{b}	0.34 ± 0.02 ^{b}	0.23 ± 0.01 ^{c}	0.07 ± 0.01 ^{c}	0.01 ± 0.01 ^{d}	0.00 ± 0.00 ^{d}
D-Sucrose	5.76 ± 0.21 ^{a}	5.12 ± 0.06 ^{b}	4.00 ± 0.11 ^{c}	3.76 ± 0.12 ^{c}	2.94 ± 0.03 ^{d}	0.75 ± 0.02 ^{e}	0.42 ± 0.03 ^{f}
D-Raffinose	0.52 ± 0.03 ^{a}	0.53 ± 0.01 ^{a}	0.59 ± 0.02 ^{a}	0.59 ± 0.02 ^{a}	0.52 ± 0.05 ^{a}	0.51 ± 0.02 ^{a}	0.54 ± 0.04 ^{a}
D-Stachyose	3.80 ± 0.19 ^{a}	3.48 ± 0.15 ^{a}	3.54 ± 0.11 ^{a}	3.85 ± 0.10 ^{a}	3.61 ± 0.45 ^{a}	4.03 ± 0.12 ^{a}	3.78 ± 0.02 ^{a}

Different lowercase letters (a–f) in each row indicate significant differences ($p < 0.05$).

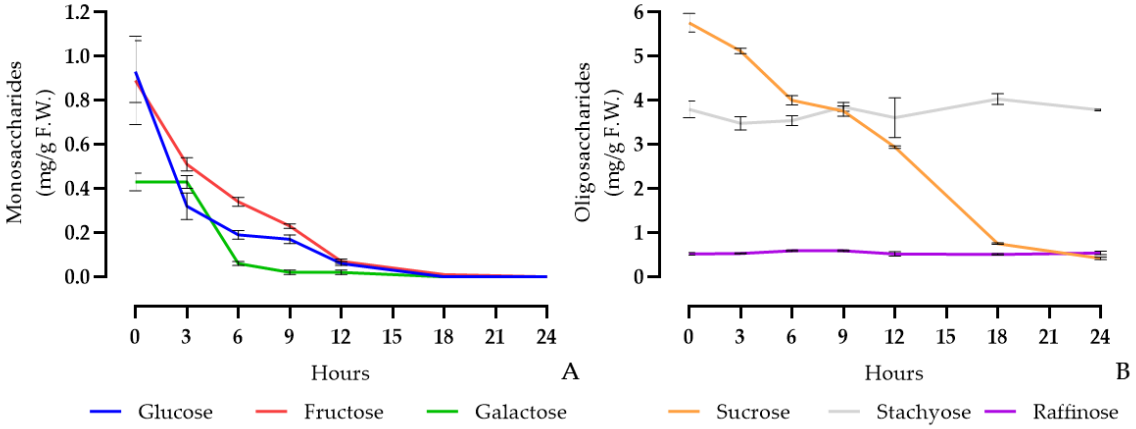

Figure 4.4 Quantitative changes in sugars during fermentation of soymilk using *Lp. plantarum* LP95 as starter: glucose, fructose galactose (A); sucrose, stachyose and raffinose (B). Error bars indicate the standard deviation of the mean (n = 3).

4.5.4 Bioconversion of isoflavones

Soymilk fermentation, using *Lp. plantarum* LP95 as a starter, significantly increased isoflavones in the aglycone-form. In detail, as reported in Table 4.4, the glycosylated isoflavones in unfermented soymilk, were daidzin, glycitin, and genistin (0.11 ± 0.01, 0.27 ± 0.05, 0.30 ± 0.01 µmol/g D.W., respectively), while their aglycosylated forms were daidzein, glycitein, and genistein (0.25 ± 0.03, 0.03 ± 0.01, 0.21 ± 0.03 µmol/g D.W., respectively). However, as showed in Figure 4.5, the content of daidzein, glycitein, and genistein increased in fermented soymilk (after 24 hours), while they remained unchanged in tofu (after 21 days), reaching the highest levels of 0.50 ± 0.01, 0.09 ± 0.01 and 0.50 ± 0.01 µmol/g D.W., respectively.

Table 4.4 Aglycone isoflavones (daidzein, glycitein, and genistein) in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter. All values are expressed as mean ± standard deviation (n = 3).

Isoflavone aglycones (µmol/g D.W.)	Unfermented soymilk	Fermented soymilk	Tofu
Daidzein	0.25 ± 0.03 ^{b}	0.50 ± 0.01 ^{a}	0.49 ± 0.02 ^{a}
Glycitein	0.03 ± 0.01 ^{b}	0.09 ± 0.01 ^{a}	0.09 ± 0.01 ^{a}
Genistein	0.21 ± 0.03 ^{b}	0.50 ± 0.01 ^{a}	0.49 ± 0.02 ^{a}

Different lowercase letters (a–b) in each row indicate significant differences ($p < 0.05$).

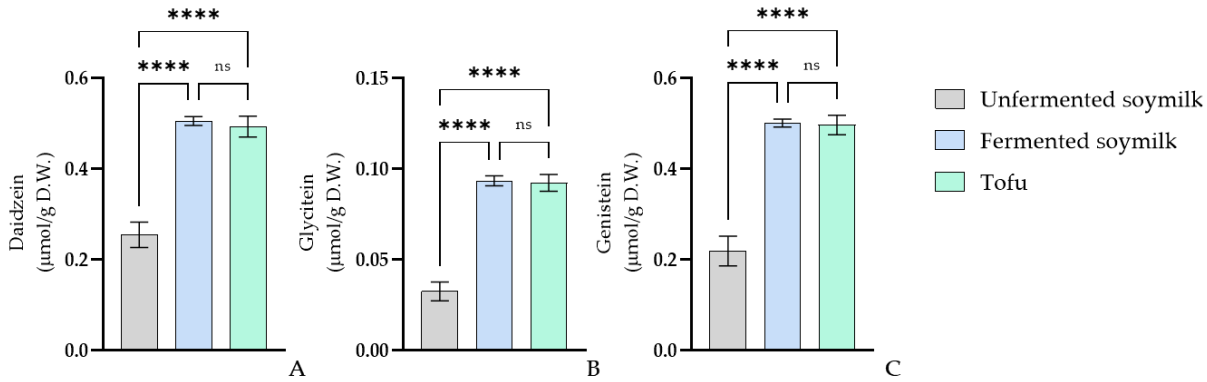

Figure 4.5 Aglycone isoflavones (A: daidzein; B: glycitein; C: genistein) in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter. Error bars indicate the standard deviation of the mean (n = 3). ns: not significant, ****: $p < 0.0001$.

4.5.5 Free amino acid profiles

Twenty-two free L-amino acids were detected and quantified in the unfermented, fermented soymilk (after 24 hours), and tofu (after 21 days). The results have been reported in Table 4.5 and representative chromatograms were showed in Figure 4.6. In particular a global consumption and subsequent decreased content of amino acids after soymilk fermentation was observed. In the unfermented soymilk, the most abundant amino acids, were γ-aminobutyric acid (GABA; 8.21 ± 1.53), arginine (6.01 ± 1.23), proline (5.60 ± 0.49), and threonine (4.67 ± 0.90). After the fermentation of soymilk, no significant variation was observed in the amounts of arginine, γ-aminobutyric acid, histidine, lysine, monoethanolamine, ornithine, and threonine. After maturation phase of Tofu (21 days) the content of the amino acids did not undergo significant changes compared to fermented soymilk except for a significant decrease in glutamine and proline.

Table 4.5 L-amino acids content in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter. All values are expressed as the mean ± standard deviation (n = 3).

L-amino acids (µmol/g D.W.)	Unfermented soymilk	Fermented soymilk	Tofu
Alanine	3.03 ± 0.52 [a]	0.50 ± 0.06 [b]	0.64 ± 0.09 [b]
Arginine	6.01 ± 1.23 [a]	7.11 ± 1.11 [a]	4.93± 1.05 [a]
Asparagine	10.8 ± 1.62 [a]	2.91 ± 0.42 [b]	2.18 ± 0.39 [b]
Aspartic Acid	1.20 ± 0.20 [a]	0.07 ± 0.01 [b]	0.08 ± 0.01 [b]
γ-aminobutyric acid	8.21 ± 1.53 [a]	8.87 ± 1.65 [a]	6.75 ± 1.16 [a]
Glutamine	0.15 ± 0.01 [a]	0.12 ± 0.03 [a]	0.08 ± 0.04 [b]
Glutamic Acid	2.73 ± 0.43 [a]	0.00 ± 0.01 [b]	0.00 ± 0.01 [b]
Glycine	0.80 ± 0.11 [a]	0.28 ± 0.11 [b]	0.22 ± 0.09 [b]
Histidine	0.95 ± 0.15 [a]	1.02 ± 0.23 [a]	0.78 ± 0.20 [a]
Isoleucine	0.85 ± 0.13 [a]	0.09 ± 0.01 [b]	0.07 ± 0.01 [b]
Leucine	1.33 ± 0.21 [a]	0.15 ± 0.02 [b]	0.13 ± 0.02 [b]
Lysine	0.76 ± 0.14 [a]	1.13 ± 0.41 [a]	0.98 ± 0.29 [a]
Monoethanolamine	0.84 ± 0.15 [a]	0.96 ± 0.16 [a]	0.79 ± 0.12 [a]
Methionine	0.32 ± 0.04 [a]	0.10 ± 0.02 [b]	0.07 ± 0.01 [b]
Ornithine	0.45 ± 0.05 [a]	0.59 ± 0.04 [a]	0.48 ± 0.13 [a]
Phenylalanine	1.17 ± 0.20 [a]	0.02 ± 0.01 [b]	0.02 ± 0.01 [b]
Proline	5.60 ± 0.49 [a]	5.70 ± 0.85 [a]	3.85 ± 1.00 [b]
Serine	1.05 ± 0.09 [a]	0.17 ± 0.03 [b]	0.15 ± 0.03 [b]
Threonine	4.67 ± 0.90 [a]	5.74 ± 1.10 [a]	4.46 ± 0.71 [a]
Tryptophan	1.65 ± 0.29 [a]	0.15 ± 0.04 [b]	0.13 ± 0.04 [b]
Tyrosine	0.93 ± 0.13 [a]	0.02 ± 0.01 [b]	0.01 ± 0.00 [b]
Valine	1.36 ± 0.20 [a]	0.08 ± 0.01 [b]	0.06 ± 0.01 [b]

Different lowercase letters (a–b) in each row indicate significant differences ($p < 0.05$).

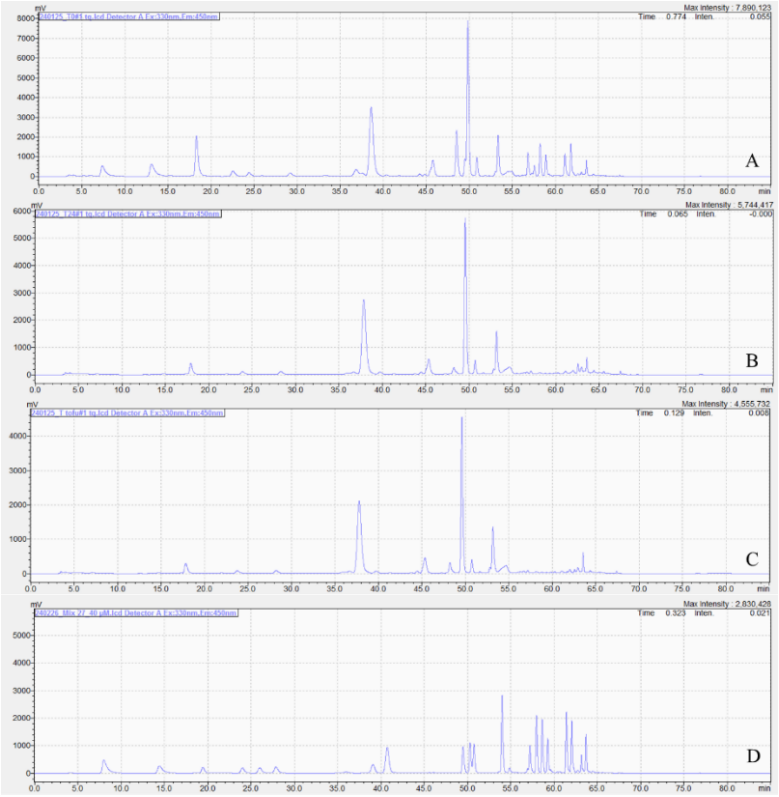

Figure 4.6 Representative chromatograms of amino acids detected via HPLC in unfermented soymilk (A), fermented soymilk (B) after 24 hours using *Lp. plantarum* LP95 as starter, and tofu after 21 days of storage (C), compared to standard mix (D).

4.5.6 Antioxidant activity

To evaluate the antioxidant activity in unfermented soymilk, fermented soymilk (after 24 hours) and tofu samples (after 21 days), were assayed by applying two different methods, the ABTS and DPPH, which are colorimetric assays where the radicals decolorize in the presence of antioxidant compounds. The two methods applied showed differences in the sensitivity of identification of the antioxidant compounds: ABTS radical scavenging activity progressively decreased, while the DPPH radical scavenging activity increased over fermentation time, as reported in Figure 4.7 and Table 4.6. In detail, the ABTS assay showed a significant decrease of TEAC specifically expressed as mg Trolox Eq./g D.W. among the unfermented soymilk (0.69 ± 0.02), fermented soymilk (0.63 ± 0.02) and tofu (0.48 ± 0.01) samples (Figure 4.7A). The DPPH assays showed a significant increase of TEAC (mg Trolox Eq./g of D.W.) during fermentation, respect to unfermented soymilk (0.13 ± 0.02) with no significant differences between fermented soymilk (0.18 ± 0.01) and tofu (0.17 ± 0.01) (Figure 4.7B).

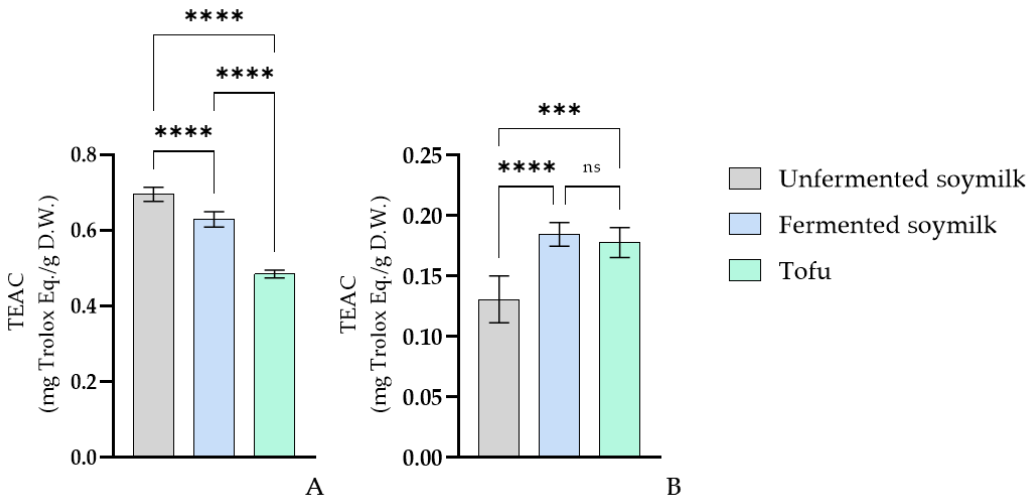

Figure 4.7 Analysis of total antioxidant activity in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter, by ABTS (A) and DPPH (B) assay. Error bars indicate the standard deviation of the mean (n = 3). ns: not significant, ***: $p = 0.0001$, ****: $p < 0.0001$.

4.5.7 Total phenolic content

The total phenolic content (TPC) of the unfermented soymilk, fermented soymilk (after 24 hours), and tofu samples (after 21 days) were reported in Table 4.6 and showed in Figure 4.8. In particular, significant differences among the samples were assigned and the measured values of TPC were 8.27 ± 0.15, 9.95 ± 0.19, 9.63 ± 0.26 mg GA Eq./g D.W. respectively.

Table 4.6 Antioxidant activity and total phenolic content in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter. All values are expressed as the mean ± standard deviation (n = 3).

	Unfermented soymilk	**Fermented soymilk**	**Tofu**
*ABTS	0.69 ± 0.02 [a]	0.63 ± 0.02 [b]	0.48 ± 0.01 [c]
*DPPH	0.13 ± 0.02 [b]	0.18 ± 0.01 [a]	0.17 ± 0.01 [a]
**TPC	8.27 ± 0.15 [c]	9.95 ± 0.19 [a]	9.63 ± 0.26 [b]

as *(mg Trolox Eq./g D.W.) ; **(mg Gallic acid Eq./g D.W.).
Different lowercase letters (a–c) in each row indicate significant differences ($p < 0.05$).

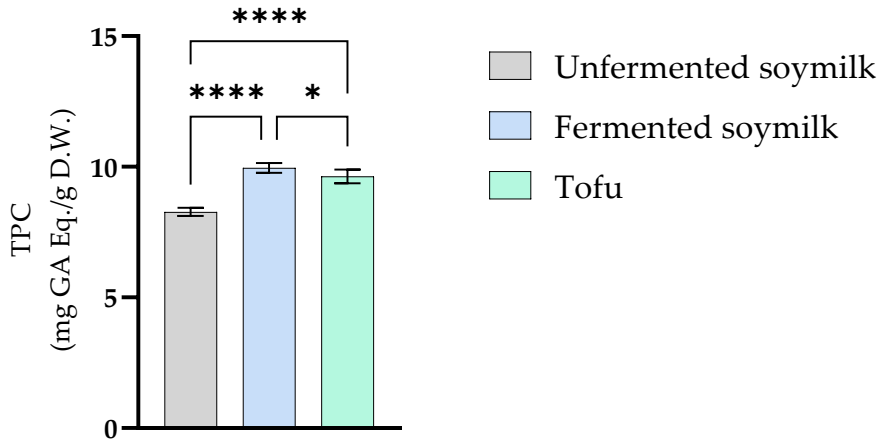

Figure 4.8 Variations of total phenolic content (TPC) expressed as mg GA Eq./g D.W. in unfermented soymilk, fermented soymilk (24 hours) and tofu (21 days) using *Lp. plantarum* LP95 as starter. Error bars indicate the standard deviation of the mean (n = 3). *: $p < 0.05$, ****: $p < 0.0001$.

4.6 Discussion

Tofu (soybean curd), a classic Asian dish, has been listed as a worldwide eaten soy food (Wang et al. 2020). Tofu is considered a well-balanced food due to its high concentration of essential components including protein, vitamins, and minerals, as well as biologically active substances like peptides, fatty acids, and isoflavones (Cui et al. 2023). Soybean tofu components are associated with the cultivar of their seeds and their genotype, composition, growing environment, and storage conditions (Zheng et al. 2020). The process of making tofu involves coagulating soymilk with salts, acids and enzymes (Li et al. 2017). Protein, sucrose, stachyose, raffinose, isoflavones, and saponins were among the many beneficial and healthy compounds present in

soymilk and the by-product (soy serum) during tofu production (Dai et al. 2021). Moreover, temperature adjustment has proven to be a successful strategy for raising the qualitative attributes of fermented products. Fermentation temperature influences the metabolites of the microbial community, which in turn affects the physicochemical properties and flavor of fermented products (Dai et al. 2023). This optimization increases the shelf life of the product, increases nutrient availability, generates bioactive molecules, eliminates hazardous chemicals from food derived from plants and extend shelf life (Liu et al. 2022). Notwithstanding the benefits, many challenges are impeding the industrialization of plant fermentation. Especially the difficulty of obtaining microorganisms appropriate for use in the fermentation of plant-based products is one of the mainly contributing reasons (Yamanaka et al. 2019; Liu et al. 2022). Given the diversity of carbon and nitrogen sources and the abundance of different organic acids and polyphenols, the strains used in the fermentation of plant-derived foodstuffs should exhibit a high diversity of metabolic pathways and a good adaptation to various grow substrates (Wuyts et al. 2020; Liu et al. 2022).

As reported by several studies on the use of *Lp. plantarum* as a starter for plant-based fermentation (Aziz et al. 2023; Guan et al. 2023; Zhang et al. 2023), it represents one of the most adaptable LAB strains, able to thrive at a wide range of temperatures and pH (Fiocco et al. 2010), and producing a variety of metabolites with distinct sensory qualities (Yilmaz et al. 2022). Therefore, *Lp. plantarum* LP95 was proposed as starter culture for soymilk fermentation due to its higher adaptability under the hostile conditions (Letizia et al. 2023).

Indeed, *Lp. plantarum* LP95 showed a marked adaptability to soymilk substrate and good fermentation kinetics towards the sugars naturally present in soymilk. In fact, after 24 hours of fermentation it was able to increase by about 1.5 log CFU/mL and lower the pH to nearly 4. Of note, the capability to catabolize different sugars in soymilk is strictly dependent on the LABs strain used as starter (Li et al. 2012). As above reported, *Lp. plantarum* LP95 is able to ferment sucrose, the main disaccharide in soymilk. In fact, after 24 hour of fermentation the residual sucrose content was approximately 7 %. However, also the content of glucose, galactose, and fructose greatly decreases during the fermentation, suggesting that this LAB shows a rapid ability to ferment completely glucose, galactose, and fructose. Furthermore, it should be emphasized that fermentation using *Lp. plantarum* LP95 as starter determine a complete fermentation of the galactose (C-4 epimer of glucose), which is a natural reducing sugar, that when administered by the diet, can directly be absorbed after digestion and enter the bloodstream to reach the target tissues, where it fuels energy production, glycosylation and other important metabolic functions (Conte et al. 2021). However, it has been reported that an excess of D-galactose can have a deleterious effects in murine models (Li et al. 2021), causing increased metabolic rates in the body

and more oxidative accumulation of free radicals, thus leading to DNA, proteins, and cell membrane lipids damage, resulting in mitochondrial damage, nerve damage and increased cognitive decline, thereby making the body age (Fatemi et al. 2018). D-galactose-induced premature aging animal models are similar to natural aging in terms of physiological and biochemical indicators, so they are widely used in drug screens and evaluations of anti-aging food products (Sadigh-Eteghad et al. 2017). Regarding the fermentation of soy oligosaccharides, the *Lp. plantarum* LP95 strain was found to be unable to metabolize soy oligosaccharides (stachyose and raffinose). Noteworthy, these oligosaccharides act as prebiotics for the colonization of other probiotic species beneficial to human health. Moreover, galactooligosaccharides (GOS) have garnered significant interest recently due to their capacity to serve as a suitable food source for bifidobacteria in the intestine and to increase the stability of beneficial bacteria in the gut, both of which can improve human health (Florowska et al. 2016; Mustafa et al. 2020). In addition the consumption of eating fermented plant-based foods alters the bacterial ecosystem in the gut by raising the number of helpful bacteria and lowering the numbers of pathogens (Cheng et al. 2005). This means that tofu produced from *Lp. plantarum* LP95 fermented soymilk will be low in monosaccharides and disaccharides, improving digestibility and the nutritional value, which makes it a possible functional low-sugar product suitable for athletes and sport people.

Previous works have reported that the fermentation of soymilk with LAB promotes the formation of bioactive compounds, like peptides, total isoflavone aglycone, γ-aminobutyric acid, and phenols (Li et al. 2012). Moreover, a positive relationship between *Lactobacillus* growth rate and isoflavone conversion has been shown, since growth rate is related to β-glucosidase activity, which converts isoflavone glycosides into aglycones (Moraes Filho et al. 2016). As it has been reported in this study, β-glucosidase activity of *Lp. plantarum* LP95 increased the content of isoflavones in aglycon form compared to unfermented soymilk. In particular, the levels of daidzein, genistein, and glycitein detected in the fermented soymilk and tofu samples were higher than in the unfermented by 50, 65 and 56 % respectively and remained unchanged until 21th day. The detection of isoflavones after 21 days strengthens the suitability of the *Lp. plantarum* LP95 strain to be used as a starter, as it does not further catabolize isoflavone aglycones. Moreover, being a kind of phenolic compounds, isoflavones were related to the main nonvolatile off-flavor in soy products, because they contribute to the bitter taste and astringent mouthfeel. In addition, aglycones have much more unpleasant flavors than glucosides (Zhu et al. 2019). Biologically, the isoflavones, which bind to nuclear estrogen receptors and exhibit estrogenic activity due to their similarity to 17β-estradiol, can enhance bone formation by stimulating osteoblastic activity primarily through i) the activation of estrogen receptors and ii) the promotion of insulin-like

growth factor-I (IGF-I) production (Arjmandi and Smith 2002; Gómez-Zorita et al. 2020; Chadha et al. 2017). Due to the resemblance to estrogen of the isoflavone molecule, it has been proposed that it can also lower the risk of cancer and cardiovascular illnesses (Gómez-Zorita et al. 2020). Furthermore, isoflavones have the ability to inhibit the activity of aromatase, the enzyme that converts testosterone into estrogen (Gómez-Zorita et al. 2020).

GABA is a non-protein amino acid, widely distributed in an extensive variety of organisms including algae, bacteria, fungi, animals, plants (Letizia et al. 2022), with established physiological functions, including neurotransmission, development of hypotension, diuretic, and tranquilizing actions. Noteworthy, the bioavailability of GABA can be enhanced by LAB fermentation, especially in the *Lactobacillus* genera (Li et al. 2012). In line with these observations, the use of *Lp. plantarum* LP95 as a starter for the production of tofu enriched in bio-functional compounds has shown that GABA levels are not significantly different in unfermented, fermented soymilk, and tofu after 21 days, making them bioavailable to the consumer.

As described above, *Lp. plantarum* LP95, after the fermentation phase, significantly increased the total phenolic content, which remained unchanged up to 21 days of storage of the tofu releasing plant phenols. Phenolic chemicals, primarily derived from plants, have been reported to exhibit anti-aging, anti-inflammatory, antioxidant, and antiproliferative properties. Nevertheless, the majority of common phenolic chemicals are usually found in insoluble forms that are difficult for humans to use when bonded with carbohydrates and proteins (Yin et al. 2020). In fact, both soluble and insoluble phenolics have been shown in numerous studies to have strong antioxidant capacities; however, the latter have a lower bioavailability than the former (Bei et al. 2018). Due to the split of these insoluble complex by enzymes released by microorganisms, such as protease, cellulase, amylase, hemicellulase, and pectinase, fermentation may promote the release of insoluble compounds, thereby increasing the functional qualities of the food (Yin et al. 2020). However, it was shown that the proteolytic activity of a number of LAB increased the amount of phenolic and isoflavones as well as the antioxidant capacity in fermented soymilk (Zhao and Shah 2014). The total antioxidant activity shows a different trend when assayed by the ABTS and DPPH methods, probably due to a different sensitivity to the various compounds with the antioxidants present in the sample. The data collected on antioxidant activity showed that the antioxidants detected by the ABTS assay decrease significantly while those detected by the DPPH assay increase significantly during fermentation. Because the ABTS assay has a higher sensitivity than the DPPH, the absolute TEAC values are highest for ABTS and lowest for DPPH. This different sensitivity were described and observed previously in other studies, not just in soymilk, but also for fruits and vegetables products (Zhang et al. 2018a; Rumpf et al. 2023).

4.7 Conclusions

Ultimately, the study elucidated the fermentation kinetics of *Lp. plantarum* LP95 in the soymilk substrate, demonstrating that it is able to rapidly ferment monosaccharides and disaccharides naturally present in soymilk after 24 hours, consequently lowering the glycemic content and leaving the GOS content unaffected. In fact, GOS act as a prebiotic substrate for the colonization of bacterial species beneficial to gut health. Furthermore, the use of *Lp. plantarum* LP95 proved useful in the production of fermented tofu, as it did not alter (degrade or bio-convert) the content of the aglyconic forms of isoflavones within 21 days, while its metabolic activity increased the content of total free phenols detected, thus increasing the amount of antioxidant compounds with putative beneficial effects on human health.

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Final remarks

Research activities carried out during this PhD thesis allowed the selection of the bacterial strain *Lp. plantarum* LP95, showing several physiological and metabolic properties that make it particularly interesting in food industry for its probiotic and pro-technological properties.

In particular, the experimental activities carried out have ascertained the applicability as a starter/probiotic culture of this LAB strain in the production of fermented soybean-based products. The preliminary *in vitro* characterization highlighted its specific enzymatic, antimicrobial and antioxidant activities. During the following experimental phases, it has been assessed that *Lp. plantarum* LP95 used as starter is able not only to ameliorate the shelf life of soymilk, but also to increase the content of bioavailable isoflavones and to maintain constant the levels of γ-aminobutyric acid **(**GABA) of the fermented product.

Noteworthy, *Lp. plantarum* LP95 has showed also to keep a high viability up to 21 days in fermented tofu, which retain a high nutritional and functional value, with unaffected levels of some amino acids and prebiotic compounds such as galactooligosaccharides (GOS).

In conclusion, the studies carried out during this PhD thesis, allowed the identification and validation of a novel LAB strain, with promising applicability in food industry, not only for its pro-technological properties, but for their putative beneficial effects on human health.

Published research articles related to PhD thesis

Letizia F, Albanese G, Testa B, et al (2022) *In Vitro* Assessment of Bio-Functional Properties from *Lactiplantibacillus plantarum* Strains. Curr Issues Mol Biol 44:2321–2334

Letizia F, Fratianni A, Cofelice M, et al (2023) Antioxidative Properties of Fermented Soymilk Using *Lactiplantibacillus plantarum* LP95. Antioxidants 12:1442

Letizia F, Fusco GM, Fratianni A, et al (2024) Application of *Lactiplantibacillus plantarum* LP95 as a Functional Starter Culture in Fermented Tofu Production. Processes 12:1093

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In Vitro Assessment of Bio-Functional Properties from *Lactiplantibacillus plantarum* Strains

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Abstract: In recent years, alongside the conventional screening procedures for the evaluation of probiotics for human usage, the pharmaceutical and food industries have encouraged scientific research towards the selection of new probiotic bacterial strains with particular functional features. Therefore, this study intended to explore novel functional properties of five *Lactiplantibacillus plantarum* strains isolated from bee bread. Specifically, antioxidant, antimicrobial and β-glucosidase activities, exopolysaccharides (EPS) production and the ability to synthesize γ-aminobutyric acid (GABA) were evaluated. The results demonstrated that the investigated *L. plantarum* strains were effective in inhibiting the growth of some human opportunistic pathogens in vitro (*Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus aureus*). Moreover, the evaluation of antioxidant and β-glucosidase activity and of EPS and GABA production, revealed a different behavior among the strains, testifying how these properties are strongly strain-dependent. This suggests that a careful selection within a given species is important in order to identify appropriate strains for specific biotechnological applications. The results highlighted that the five strains of *L. plantarum* are promising candidates for application as dietary supplements in the human diet and as microbial cultures in specific food productions.

Keywords: antioxidant activity; exopolysaccharides; *Lactiplantibacillus plantarum*; probiotic; β-glucosidase; γ-aminobutyric acid

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1. Introduction

The term 'probiotic' has been widely debated over the years, prompting a change in the generally accepted definition [1]. The current definition, formulated in 2002 by an FAO (Food and Agriculture Organization of the United Nations) and WHO (World Health Organization) working group of experts, states that probiotics are "live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [2]. This description was deemed relevant, and therefore maintained, by the Scientific Association for Probiotics and Prebiotics (ISAPP) in 2013 [3].

Besides the literal meaning, some of the criteria for microorganisms selection must be considered before their applicability as probiotics in the human diet: a probiotic agent

must show non-pathogenic properties; ability to survive in the digestive tract; adherence to the intestinal epithelium; colonization of the intestinal tract; production of antimicrobial compounds; adequate survival (stability) in the form of powder, liquid or food [3–5]. In addition to these primary characteristics, there are further functional attributes that can be contemplated in the selection of new probiotics [6–9].

Specifically, the selection of lactic acid bacteria (LAB) as probiotics possessing β-glucosidase activity and also capable of producing exopolysaccharides (EPSs) and γ-aminobutyric acid (GABA) has received much attention in recent years [10–14]. GABA (IUPAC name: 4-aminobutanoic acid) is a non-protein ubiquitous amino acid that is biosynthesized via the α-decarboxylation of glutamate, by glutamate decarboxylase (GAD) [15]. The pharmaceutical and food industries have been performing extensive studies to develop GABA-rich dietary supplements and fermented foods aiming to leverage the manifold health benefits of this amino acid, including gut modulation, neurostimulation, and cardioprotection [10,16].

About the β-glucosidase activity of LABs, it is of particular interest since it is able to increase the bioavailability of polyphenols. Several studies indicate the potential effect of polyphenols such as flavonoids and anthocyanins in reducing the incidence of cardiovascular disease, cancer, hyperlipidemias and other chronic diseases through the intake of anthocyanin-rich foods [17]. However, the effectiveness strongly depends on their bioavailability [18]. Anthocyanins contained in fruit and vegetables are bound to one or more glycosidic units. The biological activity of anthocyanins can be impaired by glycosylation, which increases their solubility in water, but on the other hand, it also makes them less reactive to free radicals and metals, thus reducing their antioxidant activity [19]. The glycosidase activity of several LABs removes the glycoside and this hydrolysis increases the bioavailability and intestinal absorption of flavonoids, which is essential for their health benefits [20]. Moreover, several LABs are also capable of producing capsular or extracellular polysaccharides (EPS), with desirable technological properties and biological activities [13,21]. EPS are widely used as viscous agents, stabilizers and emulsifiers in the food industry [22–25]. In addition, the EPS produced by LABs may be important for the survival of probiotics during gastrointestinal transit and adhesion to the intestinal wall, as well as being known to exhibit several prebiotic properties [21,22,26].

Among LABs, *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) [27] is a versatile bacterium with high adaptability to many different conditions, being isolated from several ecological niches including milk, fruit, cereal crops, vegetables, bee bread andfresh meat [28]. As with other LABs, *L. plantarum* is a common host in the gastrointestinal tracts of insects, fish and mammals, including humans [28–30]. The metabolic characteristics that are intrinsic of *L. plantarum* are the reason for their versatility and success in industrial applications, not only as a starter culture but also as a probiotic [31–33]. Furthermore, some strains of *L. plantarum* are known to produce various natural antimicrobial compounds [34,35], GABA and EPS [36–38] and to exhibit antioxidant [39,40] and β-glucosidase activity [41,42]. *L. plantarum* has the Qualified Presumption of Safety (QPS) status from the European Food Safety Authority (EFSA) and Generally Recognized As Safe (GRAS) status from the US Food and Drug Administration (US FDA) [43]. Moreover, they are microorganisms with a documented history of food use [44–46].

While a considerable number of well-characterized probiotic strains are available worldwide, research into novel strains with specific properties is still of major appeal. This is because even microorganisms within the same species show differential phenotypic characteristics [47,48]. On the basis of this scientific evidence, this work aims to evaluate some bio-functional properties of five *L. plantarum* strains, isolated from bee bread and partially characterized in previous studies [49,50]. In addition to antimicrobial, antioxidant and β-glucosidase activities, the ability of these LABs to produce EPS and GABA were evaluated. The main objective was to evaluate a possible candidacy of these *L. plantarum* strains as dietary supplements in the human diet and/or as microbial cultures for the production of functional food.

2. Materials and Methods

2.1. Lactiplantibacillus plantarum Cultures

In this investigation, five *L. plantarum* strains (LP 8, LP 25, LP 86, LP 95, and LP 100) isolated from bee bread [49] and belonging to the Food Microbiology Culture Collection of the DiAAA (Department of Agricultural, Environmental and Food Sciences, University of Molise, Campobasso, Italy) were used. 16S rDNA sequences were submitted in GeneBank [51] under accession numbers from OM033651 to OM033655 (accessed on 28 December 2021). *L. plantarum* ATCC 14917, belonging to the American Type Culture Collection (ATCC Manassas, VA, USA), was used as reference strain with proven in vitro probiotic properties [52–54].

2.2. Antimicrobial Activity

The antimicrobial activity of the *L. plantarum* strains (producers) was evaluated against the following indicator bacteria: *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 11775, *Proteus mirabilis* ATCC 29906, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853, belonging to the American Type Culture Collection (ATCC Manassas, VA, USA). The LABs and the indicator strains were grown at 37 °C in MRS and BHI broth (Oxoid Ltd., Hampshire, UK), respectively.

The *L. plantarum* strains in log phase (10^8 CFU/mL) were centrifugated (8,000 rpm for 10 min at 4 °C) and filtered (cellulose acetate membrane, pore size 0.22 µm Sigma-Aldrich, Merck KGaA, Darmstadt, GE) to obtain the cell-free supernatant (CFS). The antimicrobial activity of CFS was evaluated according to Iorizzo et al. [50] with some modifications. In brief, 20 mL of BHI soft agar (0.7% agar, *w*/*v*) inoculated with an overnight culture of indicator bacteria (final concentration of about 10^7 CFU/mL) were poured into Petri plates (Ø 90 mm). Wells of 5.0 mm in diameter were bored into the single plate and 50 µL of CFS, of each producer strain, were placed into different wells. As a control, 50 µL of MRS adjusted to pH 3.8 with hydrochloric acid 1 N (Sigma-Aldrich, Merck KGaA, Darmstadt, GE) was used. After incubation at 37 °C for 48–72 h, the plates were observed and antibacterial activity was reported as the diameter (mm) of the clear zone of inhibition (ZOI) around the inoculated wells [55].

2.3. Cell Protein Assay

For subsequent analysis (EPS and GABA production; antioxidant activity) the LAB cultures (10^8 CFU/mL), grown in the specific media reported below, were centrifugated (8000 rpm for 10 min at 4 °C) and filtered (cellulose acetate membrane, pore size 0.22 µm Sigma-Aldrich, Merck KGaA, Darmstadt, GE) to obtain the cell pellet (CP). The CP was washed twice in sterile saline solution (NaCl 0.9%, *w*/*v*), resuspended in NaOH 0.1 M according to Iorizzo et al. [56], and then subjected to three cycles of sonication (Labsonic M; Sartorius Lab Instruments GmbH & Co. KG, Goettingen, GE) at 12 W for 30 s, with 60 s of pause between the cycles to improve cellular lysis. Subsequently, the samples were centrifugated at 13,000 rpm for 5 min at 4 °C, and cell extract supernatants (CES) were used for protein quantification according to Di Martino et al. [57] using a BioSpectrometer (Eppendorf, Hamburg, GE). Total protein concentrations were calculated by means of a calibration curve using Bovine Serum Albumin (BSA) as standard. All chemicals used were supplied by Sigma-Aldrich, Merck KGaA, Darmstadt, GE.

2.4. β-Glucosidase Activity

For the assessment of β-glucosidase activity, the tested LABs were cultured in modified MRS (MRS_m) according to Ávila et al. [58]. The bacterial cultures (10 mL, 10^8 CFU/mL) were centrifugated (8000 rpm for 10 min at 4 °C) to obtain the cell pellet (CP_{GA}); they were washed in sterile saline solution (NaCl 0.9%, *w*/*v*) and resuspended in isotonic Phosphate Buffer Saline (PBS) pH 7.4. The β-glucosidase activity of the *L. plantarum* strains was performed as described by Ávila et al. [58], with some modification. Briefly, β-glucosidase reactions were performed mixing 50 µL of 20 mM *p*-nitrophenyl-β-D-glucopyranoside

(*p*-NPG) in PBS pH 6.5, with 50 µL of bacterial suspension (OD_{600} 0.5) and bringing the final volume of the solution to 1 mL with PBS. Next, 200 µL of the mix were placed in a microplate well and read at 405 nm in kinetic mode with a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific, Waltham, MA, USA) for six hours. Enzyme activity was expressed as the amount of β-glucosidase that released 1 nmol of *p*-nitrophenol (*p*-NP) per unit volume per minute: *p*-NP/(min mL). A calibration curve was obtained using *p*-nitrophenol as standard. The values of β-glucosidase activity were expressed as the ratio of enzyme units. All chemicals used were supplied by Sigma-Aldrich, Merck KGaA, Darmstadt, GE, and the tests were conducted in triplicate.

2.5. Exopolysaccharide (EPS) Production

For EPS production, LABs were grown in MRS_m. Over 48 h at 37 °C, the cultures (10^8 CFU/mL) were centrifugated (8000 rpm for 10 min at 4 °C) to obtain the cell-pellet (CP_{EPS}) and the cell-free supernatant (CFS_{EPS}).

The CP_{EPS} and CFS_{EPS} were used for the determination of EPS-b (bound exopolysaccharides) and EPS-r (released exopolysaccharides), respectively, as described by Tallon et al. [59]. EPS quantification was performed by the anthrone-sulfuric acid colorimetric assay as reported by Laurentin et al. [60]. The culture medium without bacterial inoculum was used as control. The EPS-r values are expressed as µmol glucose Eq./mL, while the absolute concentrations of EPS-b* are expressed as nmol glucose Eq./mL. Instead, EPS-b values related to cellular protein content (EPS-b**) were expressed as the ratio between nmol glucose Eq. and cell proteins (µg BSA Eq.). The cell proteins were determined as described in Section 2.3.

The tests were conducted in triplicate. All chemicals used were supplied by Sigma-Aldrich, Merck KGaA, Darmstadt, GE.

2.6. Antioxidant Activity (ABTS Assay)

LAB cultures (10 mL) in MRS_m, over 48 h at 37 °C, were centrifugated (8000 rpm for 10 min at 4 °C) at their log phase (10^8 CFU/mL) to obtain the cell pellet (CP_{AA}). The CP_{AA} were washed twice in sterile saline (NaCl 0.9%, *w*/*v*), resuspended in cold pure methanol (1 mL), and subjected to three cycles of sonication (Labsonic M; Sartorius Lab Instruments GmbH & Co. KG, Goettingen, GE at 12 W for 30 s, with 60 s of pause between the cycles to improve cellular lysis. Subsequently, the samples were centrifugated at 13,000 rpm for 5 min at 4 °C, and cell extract supernatants (CES_{AA}) were used to evaluate total antioxidant activity (TAA), using the 2,2 azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS+) radical cation assay according to Re et al. [61], with some modifications. Briefly, ABTS was dissolved in pure methanol to a concentration of 7 mM. ABTS radical cations (ABTS+) were produced by reacting the ABTS methanol solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 24 h before use. The ABTS·+ solution was diluted with cold pure methanol to an optical density (OD) of 0.700 at 745 nm. Then, 100 µL of CES_{AA} were mixed with 900 µL of the ABTS·+ solution. The OD was measured at 745 nm after 6 min in the dark at room temperature using a BioSpectrometer (Eppendorf, Hamburg, GE). Trolox was used as the standard for the calibration curve.

The antioxidant activity was expressed as the ratio (*w*/*w*) between µg/mL Trolox and mg/mL of cell proteins (BSA Eq.) [62]. The cell proteins were determined as described in paragraph 2.3. All the chemical compounds used were supplied by Sigma-Aldrich, Merck KGaA Darmstadt, GE and the tests were conducted in triplicate.

2.7. γ-Aminobutyric Acid (GABA) Production

For the GABA production assay, the *L. plantarum* strains were grown at 37 °C in LFM medium, whose composition is reported in Table S1 (Supplementary Material). The broth cultures (10 mL; 10^6 CFU/mL) were centrifugated to obtain the cell pellet (CP_{LFM}). The CP_{LFM} were washed twice in sterile saline solution (NaCl 0.9%, *w*/*v*), resuspended in 600 µL

of ethanol/water (40/60, *v*/*v*), and then subjected to three cycles of sonication (Labsonic M; Sartorius Lab Instruments GmbH & Co. KG, Goettingen, GE Sartorius Lab Instruments GmbH & Co. KG, Goettingen, GE) at 12 W for 30 s, with 60 s of pause between the cycles to improve cellular lysis. Subsequently, the samples were centrifugated at 13,000 rpm for 5 min at 4 °C and cell extract supernatants (CES_{LFM}) were used for GABA quantification via High Performance Liquid Chromatography (HPLC) using a Hewlett Packard 1100 (Agilent, Santa Clara, CA, USA) equipped with Degasser G1322A, Binary Pump G1312A, Autosampler ALS G1313A, Thermostatted Column Compartment G1316A, DAD Detector G1315B and Fluorescence Detector G1321A.

For the automatic pre-column derivatization, 20 µL of CES_{LFM} were added to 40 µL of *o*-Phthalaldehyde (OPA) reagent (Agilent, Santa Clara, CA, USA). Subsequently, 30 µL of OPA-derivatives mix were injected into a reverse-phase column (ZORBAX Eclipse Plus C18, 4.6 × 250 mm 5 µm; Agilent, Santa Clara, CA, USA), protected with SecurityGuard Phenomenex (Torrance, CA, USA). The elution was performed at a flow rate of 0.85 mL/min at 27 °C using a solution of sodium acetate buffer 50 mM + tetrahydrofuran 0.3% + methanol 20% pH 6.4 and methanol 100%, as described by Carillo et al. [63]. The fluorescence of OPA derivatives was detected at 340 nm excitation and 450 nm emission wavelengths. The GABA identification and quantification were carried out using HP ChemStation software ver. A.06.03 (Agilent, Santa Clara, CA, USA), compared to retention times and internal peak area of reference standards. The data were expressed as ratio between pmol of GABA and cell proteins content (µg BSA Eq.). The cell proteins were determined as described in paragraph 2.3. All chemicals standards used were supplied by Sigma-Aldrich, Merck KGaA, Darmstadt, GE and the tests were conducted in triplicate.

2.8. Statistical Analysis

Experiments were performed in triplicate (n = 3) and all data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed by analysis of variance (ANOVA) followed by Tuckey's multiple comparisons. Statistical significance was set to p-values < 0.05. The software SPSS Statistics 21 (IBM Corp, Armonk, NY, USA) was used for the analysis.

3. Results

3.1. Antimicrobial Activity

The numerical data of the antimicrobial activity, estimated as clear zone of inhibition ZOI (mm), exhibited by LABs against various indicator bacteria are presented in Table S2 (Supplementary Material). In Figure 1, the results are shown as inhibition rate (%). More specifically, no antimicrobial activity was observed when MRS pH 3.8 was used, while the six tested *L. plantarum* strains presented different inhibitory activities against the five indicator bacteria. The cell free supernatant (CFS) of all six *L. plantarum* strains inhibited the development of *P. aeruginosa* and *E. faecalis*. With the exception of LP 25, the other LAB strains were effective against *E. coli* and *P. mirabilis*. The *L. plantarum* LP 86, LP 95, LP 100 and ATCC 14917 strains were effective in counteracting the growth of *S. aureus*. The highest antagonistic activity of the tested *L. plantarum* strains was against *P. aeruginosa* with a ZOI between 18 (LP 100) and 24 mm (LP 8).

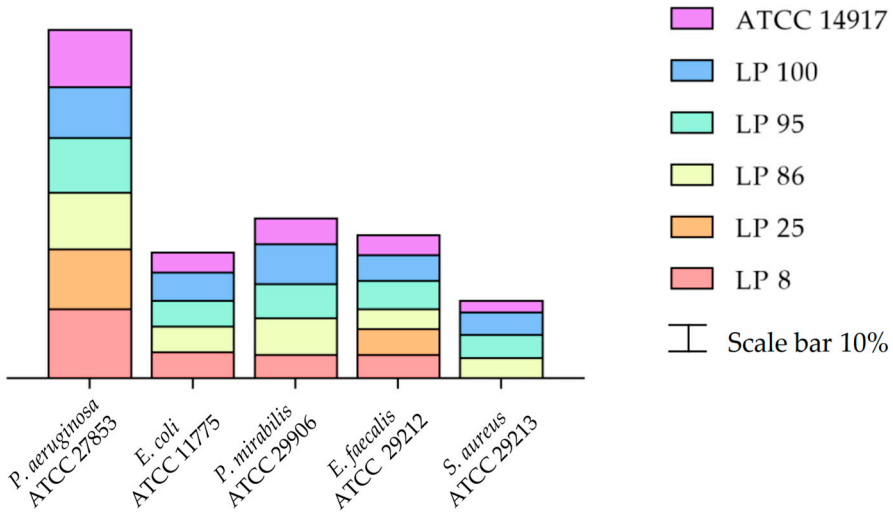

Figure 1. Antimicrobial activity of the *L. plantarum* strains CFS against different indicator bacteria expressed as inhibition rate (%).

3.2. EPS Production

The values of EPS production were reported in Table 1. In detail, the EPS-r values ranged from 2.84 (LP 25) to 3.43 µmol glucose Eq./mL (LP 8), while the values of EPS-b* varied from 38.95 (LP 86) to 223.42 nmol glucose Eq./mL (LP8). Lastly, about the EPS-b** content, the range of values was between a minimum of 0.016 (LP 86) and a maximum of 0.069 (LP 95) nmol glucose Eq./µg BSA Eq. (bovine serum albumin).

Table 1. EPS-released (EPS-r), EPS-bound (EPS-b) and EPS-bound related to cell proteins (EPS-b**). All values are expressed as mean ± standard deviation (n = 3). Different lowercase letters (a–d) in each row indicate significant differences ($p < 0.05$).

	***L. plantarum* Strains**					
EPS Fractions	**LP 8**	**LP 25**	**LP 86**	**LP 95**	**LP 100**	**ATCC 14917**
EPS-r	3.43 ± 0.08 ^{a}	2.84 ± 0.15 ^{b}	3.29 ± 0.20 ^{a}	3.06 ± 0.17 ^{a}	3.03 ± 0.18 ^{a}	3.16 ± 0.13 ^{a}
EPS-b*	135.91 ± 5.67 ^{c}	136.23 ± 5.08 ^{c}	38.95 ± 1.91 ^{d}	223.42 ± 7.90 ^{a}	160.96 ± 7.94 ^{b}	51.45 ± 2.74 ^{d}
EPS-b**	0.048 ± 0.007 ^{b}	0.046 ± 0.005 ^{b}	0.016 ± 0.004 ^{c}	0.069 ± 0.011 ^{a}	0.050 ± 0.004 ^{b}	0.026 ± 0.006 ^{c}

The data are expressed as follows. EPS-r: µmol glucose Eq./mL; EPS-b*: nmol glucose Eq./mL; EPS-b**: nmol glucose Eq./µg BSA Eq.

3.3. ABTS Antioxidant Activity

The ABTS radical-scavenging assay has been used to assess the ability of metabolic compounds produced by the different *L. plantarum* strains to act as free radical scavengers or hydrogen donors. The Trolox equivalent antioxidant capacity is expressed with respect to cellular protein content. As reported in Table 2, antioxidant activity values are significantly different among the tested strains. In detail, the strains LP 95 and LP 100 exhibited the highest values, which amounted to 14.23 and 15.84 µg Trolox Eq./mg BSA Eq., respectively, while the lowest value has been found for strain LP 86 (3.89 µg Trolox Eq./mg BSA Eq.).

Table 2. Antioxidant and β-glucosidase activities of *L. plantarum* strains. All values are expressed as mean ± standard deviation (n = 3). Different lowercase letters (a–c) in each row indicate significant differences ($p < 0.05$).

	***L. plantarum* Strains**					
	LP 8	**LP 25**	**LP 86**	**LP 95**	**LP 100**	**ATCC 14917**
*** Antioxidant activity**	6.46 ± 0.65 ^{c}	6.51 ± 0.38 ^{c}	3.89 ± 0.34 ^{d}	14.23 ± 1.60 ^{a}	15.84 ± 0.80 ^{a}	13.35 ± 0.26 ^{b}
**** β-glucosidase activity**	0.30 ± 0.06 ^{a}	0.39 ± 0.07 ^{a}	0.38 ± 0.08 ^{a}	0.45 ± 0.03 ^{a}	0.26 ± 0.08 ^{b}	0.26 ± 0.08 ^{b}

* Expressed as μg Trolox Eq./mg BSA Eq.; ** Expressed as nmol *p*-Nitrophenol/(min mL).

3.4. β-Glucosidase Activity

The enzymatic activity exhibited by the bacterial cultures was assessed as the release of *p*-NP from the *p*-NPG substrate in the time unit. As reported in Table 2, all tested strains showed β-glucosidase activity, which ranged from 0.26 to 0.45 nmol *p*-NP/(min mL). The data show significant differences in this specific enzymatic activity among the strains. The minimum values were recorded for LP 100 and ATCC 14917 strains, whereas the highest value corresponded to *L. plantarum* LP 95.

3.5. GABA Production

The biosynthetic ability of *L. plantarum* strains to produce GABA was assessed in LFM medium with and without L-glutamic acid (GLU). GABA contents were determined by injecting an aliquot of the cell extract supernatants (CES_{LFM}) via HPLC after precolumn OPA derivatization, and the amounts were calculated as the internal peak area. Representative chromatograms are shown in Figure 2. The results, reported in Table S3 and summarized in Figure 3, were expressed as the ratio between the pmol of GABA and the cell proteins content (μg BSA Eq.) The GABA-specific contents in the CES_{LFM} obtained from the six *L. plantarum* strains examined showed significant differences as shown in Figure 3. Values varied within a range of 0.345 (LP 25) and 2.27 μg Trolox Eq./mg BSA Eq. (LP 95).

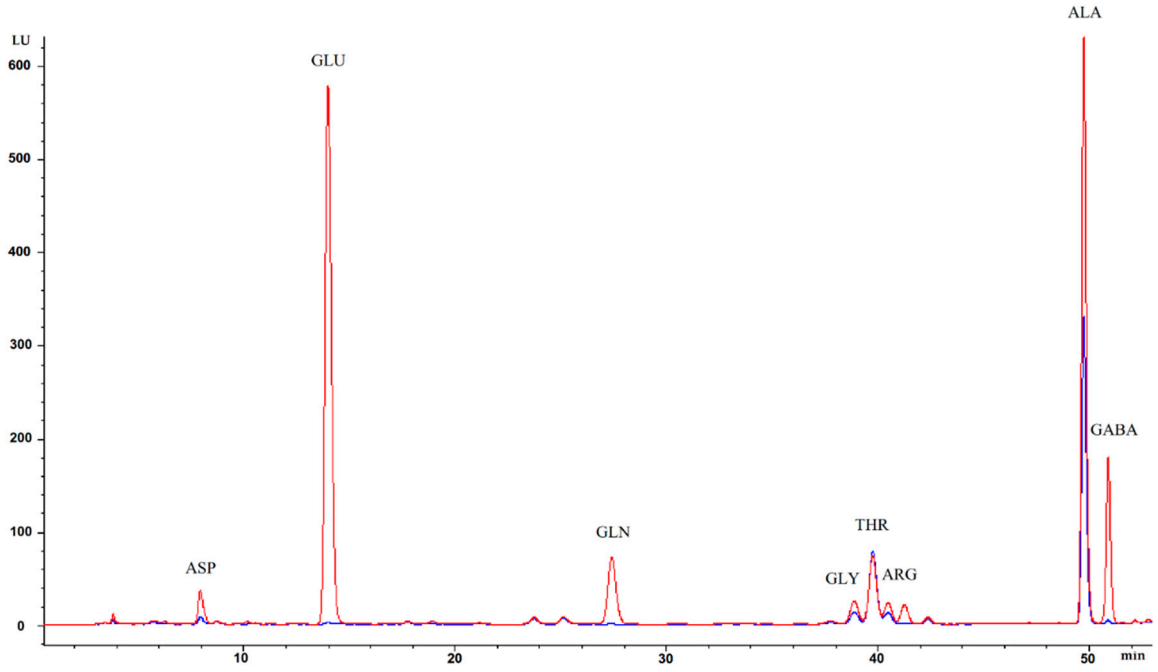

Figure 2. GABA production from *L. plantarum* LP 95 in LFM medium with (■) and without (■) L-glutamic acid (GLU).

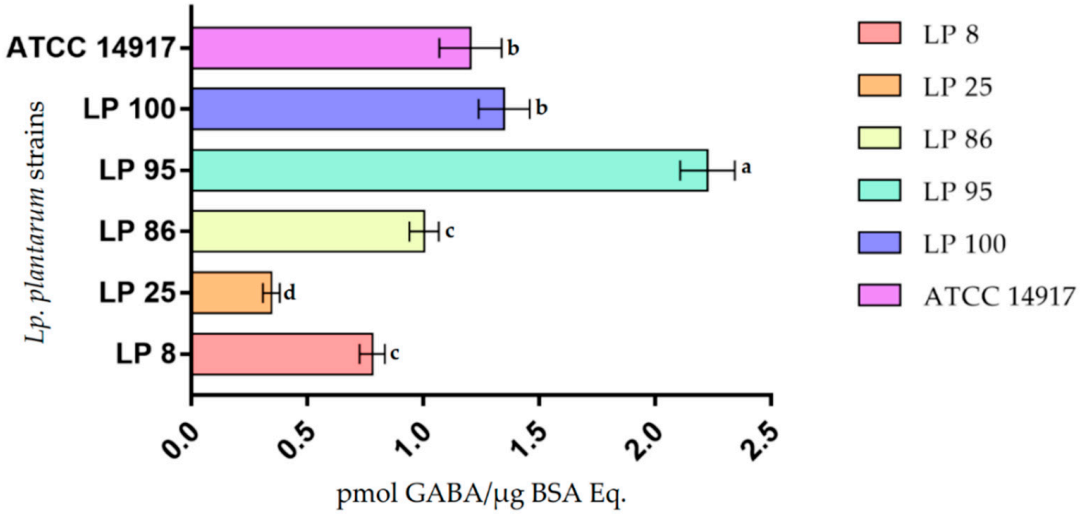

Figure 3. GABA amounts synthesized from *L. plantarum* strains grown in LFM medium with L-glutamic acid (GLU) (100 µM). All data are expressed as mean ± standard deviation ($n = 3$). Different lowercase letters (a–d) indicate significant differences ($p < 0.05$).

4. Discussion

The characterization of microorganisms beneficial for food preservation and the prevention of pathogen growth is mainly based on their ability to produce bioactive compounds that have antimicrobial activity.

In our investigation the reference strain ATCC 14917 and the five *L. plantarum* isolated by us from bee bread [49,50] were able to limit the growth of indicator bacteria, but with varying degrees of intensity. The mechanisms underlying the inhibitory capacity of LABs are attributable to multiple mechanisms of action, such as individual compounds that may cause membrane destabilization (such as fatty acids or peptides), H^+ gradient interference (such as organic acids or peptides), or enzyme inhibition (such as hydroxy acids) [64]. Moreover, there may be some synergistic and/or additive effects that involve various compounds [65].

Since no effect on the growth of indicator bacteria was observed in our study using MRS pH 3.8, the inhibition of the LABs against pathogens cannot be ascribed to a simple consequence of the lowering of pH due to the bacterial metabolism of the producer strains. Antimicrobial activity, therefore, was most likely caused by the production of organic acids, protein compounds or other antimicrobial metabolites contained in CFS, which need to be further investigated.

The LAB food-grade EPSs have earned considerable attention for their structural and sensory properties in the food industry, primarily for dairy products and more recently for the improvement of cereal-based products [37,66–72]. Furthermore, EPSs have also gained major relevance in pharmacological and nutraceutical applications, as they can serve as immunomodulatory, antioxidant, anti-cancer, cholesterol-lowering, antimicrobial and prebiotic agents [73–77]. Microbial EPSs are not permanently anchored to the microbial cell surface and exist in two forms based on their localization: cell-bound EPSs, which are tightly adhered to the bacterial surface (bound exopolysaccharides; EPS-b), and EPSs that are released into the surrounding medium (released exopolysaccharides; EPS-r). In our research, we quantitatively determined the two exopolysaccharide matrices and showed the ability of the tested *L. plantarum* strains to produce these compounds with significant differences in EPS-b production.

Our results confirm that several *L. plantarum* strains are good EPS producers [14,37,78]. In particular, the strains LP 95 and LP 100 produced quantities significantly higher compared to the other strains. The rheological and prebiotic properties of the EPS produced

by LAB [66] make it for possible technological applications in the food industry of the *L. plantarum* LP 95 and LP 100 strains.

Some LAB strains possess both enzymatic and non-enzymatic antioxidant activity, which reduces the risk of reactive oxygen species (ROS) accumulation during food ingestion, resulting in a decrease in oxidative stress [79–82]. Over the last decade, considerable attention has been drawn to the use of LAB as natural antioxidants in the food industry [83]. Apart from some cellular constituents (exopolysaccharides, peptidoglycan, lipoteichoic acid and proteins) LABs are known to have complex antioxidant activity, and different strains operate different mechanisms: chelation of toxic ions (Fe^{2+} and Cu^{2+}), synthesis of antioxidant compounds (e.g., glutathione, butyrate, folate) and activation of enzymatic pathways for the scavenging of free radicals [39,84–87]. It has also been reported that the antioxidant activity of LABs may be linked to some proteolytic-derived peptides and may vary depending on the species and on the strain [83].

Whereas in other studies, the antioxidant activity of various *L. plantarum* strains is associated with EPS [85,87,88].

Tang et al. [81] reported that CFS from *L. plantarum* MA2 exhibited strong reducing capacity, lipid peroxidation inhibition, Fe^{2+}-chelating and free radical scavenging activities. Moreover, the cell homogenate revealed glutathione peroxidase activity and superoxide dismutase activity.

In our work, we evaluated the antioxidant activity by means of the ABTS assay, which is considered one of the most sensitive procedures [89] and a valid method for the assessment of the antioxidant activity of both hydrophilic and lipophilic extracts [90].

All five strains of *L. plantarum* exhibited antioxidant activity, and the mechanism or molecules involved in this behavior require more detailed study.

Over recent years, the pharmaceutical and food industries' research has strongly focused on the development of dietary supplements and fermented foods enriched with GABA for the various health benefits of this amino acid, including neurostimulation and cardio protection [10,11]. GABA-producing LABs may provide new opportunities for naturally fermented functional food products [16]. LABs synthesize GABA via the GAD pathway, in which the GAD protein localized in the cytoplasm is the key enzyme. Strains with the *gad* gene are therefore able to synthesize GABA. The first step in the GAD pathway is carried out by a GLU/GABA antiporter, encoded by a *gadC* gene. This antiporter pumps the GLU precursor into the microorganism [10]. LABs have strain-specific GABA synthesis capacities and several environmental factors, such as pH and temperature, impact the expression of the *gad* gene [91,92]. *L. plantarum* is one of the most widely distributed and common bacteria occurring in fermented foods and therefore it would be valuable to achieve effective GABA production using selected strains from this species.

Our results showed that the tested *L. plantarum* strains are able to synthesize GABA, confirming that bacteria belonging to this species are able to produce this amino acid [93,94]. To assess the ability to produce GABA, we used the medium LFM (Table S1) with a defined centesimal composition to exclude interference from undefined substances in the MRS medium based on complex matrices (e.g., yeast extract and meat extract).

LABs with the ability to synthesize GABA can offer new opportunities in the design of functional foods. However, further investigations are needed to optimize the fermentation conditions and to improve the production of this non-proteic amino acid [11,95,96].

Isoflavones are plant-based polyphenolic compounds, predominantly found in glycosidic forms that can be hydrolyzed by intestinal and bacterial β-glucosidases to release aglycones, which are subsequently absorbed in the large intestine [20,41].

β-glucosidase LAB producers have a major role in the release of free flavonoids from their glycosylate precursors found in fruits and other plant tissues and enhance the bioavailability of these health-promoting plant metabolites [97].

In our study, the selected *L. plantarum* strains exhibited β-glucosidase activity without significant differences among them. As a result of this specific enzymatic activity, it would therefore be interesting to use them as starter cultures in future applications to optimize the

organoleptic, technological and nutritional properties of fermented foods (e.g., soy-derived foods or vegetable beverages) [12,97].

5. Conclusions

This in vitro study allowed us to screen some *L. plantarum* strains possessing good potential for further studies on their probiotic and functional properties.

We found a large heterogeneity related to examined properties confirming that these are strain-specific.

Therefore, our results suggest that a careful selection within a given species is of utmost importance to identify appropriate strains for specific biotechnological applications. In particular, the evaluation of our results shows that *L. plantarum* LP 95 was the best-performing strain and that could be a candidate as new a probiotic in the para-pharmaceutical sector and as food microbial culture to improve some functional characteristics of specific products.

Clearly, we consider this study a preliminary selection and further in vitro and in vivo investigations are needed for the use of this LAB in human diet.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cimb44050158/s1, Table S1: LFM medium composition; Table S2: Antimicrobial activity by cell-free supernatant of the different *L. plantarum* strains against indicator bacteria; Table S3: GABA intracellular contents of the different *L. plantarum* strains.

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Antioxidative Properties of Fermented Soymilk Using *Lactiplantibacillus plantarum* LP95

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Abstract: In recent times, there has been a growing consumer interest in replacing animal foods with alternative plant-based products. Starting from this assumption, for its functional properties, soymilk fermented with lactic acid bacteria is gaining an important position in the food industry. In the present study, soymilk was fermented with *Lactiplantibacillus plantarum* LP95 at 37 °C, without the use of stabilizers as well as thickeners and acidity regulators. We evaluated the antioxidant capacity of fermented soymilk along with its enrichment in aglycone isoflavones. The conversion of isoflavone glucosides to aglycones (genistein, glycitein, and daidzein) was analyzed together with antioxidant activity (ABTS) measurements, lipid peroxidation measurements obtained by a thiobarbituric acid reactive substance (TBARS) assay, and apparent viscosity measurements. From these investigations, soymilk fermentation using *Lp. plantarum* LP95 as a starter significantly increased isoflavones' transformation to their aglycone forms. The content of daidzein, glycitein, and genistein increased after 24 h of fermentation, reaching levels of 48.45 ± 1.30, 5.10 ± 0.16, and 56.35 ± 1.02 µmol/100 g of dry weight, respectively. Furthermore, the antioxidant activity increased after 6 h with a reduction in MDA (malondialdehyde). The apparent viscosity was found to increase after 24 h of fermentation, while it slightly decreased, starting from 21 days of storage. Based on this evidence, *Lp. plantarum* LP95 appears to be a promising candidate as a starter for fermented soymilk production.

Keywords: functional food; soymilk; fermentation; isoflavones; *Lactiplantibacillus plantarum*

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1. Introduction

The growing interest of consumers in nutritionally enriched and health-promoting foods provokes interest in the eventual development of fermented functional foods [1]. In this view, the consumption of soybean (*Glycine max*) and its products has received attention from researchers and consumers both for the nutritional value and for its potential health benefits, including reducing the risk of cardiovascular diseases, diabetes, breast cancer, and chronic inflammation, due to the presence of bioactive compounds, such as isoflavones [2,3]. The water extract of soybeans, namely, soymilk, is an important oriental traditional beverage [4], considered a colloidal system as it contains protein and minerals [5]. This beverage can be a good substitute for animal-based milk and an ideal food for vegans diets and lactose-intolerant people [6]. Despite the listed properties of soymilk, its consumption in Western countries is still limited due to its unpleasant taste and its antinutritional factor, such as phytates that compromise the natural availability of minerals, as well as the presence of indigestible oligosaccharides, such as raffinose and stachyose, that cause flatulence [7,8]. Considering these limitations, the fermentative biotransformation approach using lactic acid bacteria (LAB) is an effective strategy to improve the nutritional, textural, and rheological properties of soymilk [3]. Isoflavones

are phytochemical compounds naturally present in soymilk, and their enteric absorption is related to their chemical structure. The concentrations of the different forms of these bioactive compounds in soymilk are related to the soybean cultivar, time of soaking, the ratio of soybean to water, filtration, and thermal treatments [9,10]. The principal isoflavones of soy are daidzein, genistein, and glycitein, which exist in plants conjugated with sugars [11,12].

Glycosides can be hydrolyzed in the oral cavity [13] and, mainly, in the intestine to their aglycones, which are then absorbed in the intestine [14]. The flavonoid glycosides are considered biologically inactive, and their bioavailability requires the initial hydrolysis of the sugar moiety by intestinal β-glucosidases for uptake into the peripheral circulation [15].

Several studies have shown that it is possible to reverse the glucoside/aglycone ratio in soy food through LAB strains with β-glucosidase activity [16,17]. Therefore, the use of these bacteria as a starter, with the aforementioned enzymatic activity, in the fermentation of soymilk could contribute to increasing bioavailable isoflavones. [18].

Moreover, some LAB, when assumed through the consumption of fermented foods, show antioxidant effects, which differ among species due to different concentrations and types of antioxidants, and therefore could play a pivotal role in human health [19,20].

Among LAB species, *Lactiplantibacillus plantarum* has the Qualified Presumption of Safety (QPS) status from the European Food Safety Authority (EFSA) and the Generally Recognized as Safe (GRAS) status from the US Food and Drug Administration (US FDA) [21]. In addition, this bacterial species has a documented history as a microbial food culture [22–24]. Based on previous studies [25], in this work, we used the *Lp. plantarum* LP95 strain which possesses antimicrobial, β-glucosidase activities and exopolysaccharide (EPS) production capacity, as a starter to obtain fermented organic soymilk without the use of stabilizers, thickeners, and acidity regulators.

Therefore, the main aim of our study was to evaluate the antioxidant capacity of fermented soymilk and its enrichment in isoflavone aglycone.

Furthermore, the fermented soymilk was characterized by its flow behavior and apparent viscosity.

2. Materials and Methods

2.1. Bacterial Strain

In this study, *Lp. plantarum* LP95 (Genbank accession number: OM033654), isolated from fermented pollen (bee bread), was cultured at 37 °C in MRS medium (de Man, Rogosa and Sharpe Broth, Oxoid Ltd., Basingstoke, Hampshire, UK). After 12 h, bacterial cells were harvested by centrifugation (10,000 rpm, 5 min, 4 °C) and washed with sterile saline solution (0.9% *w*/*v* NaCl). The supernatant was discarded, and the cell pellet was recovered, resuspended in saline solution and properly diluted to adjust cell concentration before being used as the starter.

2.2. Chemicals

All chemicals were purchased from Sigma (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Eluents and water for analytical analysis were purified using a Milli-Q system (Millipore, Burlington, MA, USA).

2.3. Preparation of Soymilk

Handcrafted soymilk was prepared according to Wang et al. [26] with some modification by soaking 400 g of mature yellow organic soybeans (*Glycine max*, marketed by BV&FdL S.r.l., Bentivoglio, Italy) in 1200 mL of sterile distilled water for 12 h.

Subsequently, soybeans were drained, dried, and blended with 1400 mL of water. The resulting product was subjected to blanching (90 °C, 10 min), afterward filtered and sterilized at 121 °C for 15 min, and finally cooled for the inoculum phase. Next, the entire mass was homogenized by stirring before adding the microbial starter. After the starter

inoculation, soymilk was portioned into three different sterile glass containers (200 mL) for subsequent experimental and analytical steps.

2.4. Screening for Optimum Temperature

To optimize the fermentation phase, preliminary tests were carried out inoculating the obtained soymilk with *Lp. plantarum* LP95 at a concentration of 10^6 CFU/mL at three temperatures (20 °C, 28 °C, and 37 °C). The pH and viable cell counts were monitored at 0, 3, 6, 9, 24, 32, 48, and 72 h.

2.5. Production of Fermented Soymilk

According to the preliminary screening for the optimum temperature, the fermentation phase was conducted at 37 °C, inoculating the soymilk with *Lp. plantarum* LP95 (10^8 CFU/mL). After 24 h, the clot was broken, and the final product was stored at 4 °C. Samples were analyzed immediately and every 7 days until the 49th day.

2.6. Determination of Water-Holding Capacity (WHC)

WHC is defined as the capacity of soymilk to retain all or part of its water, and its determination was assayed according to the method described by Li et al. [5]. Briefly, fermented soymilk (30 g) was centrifuged at 8000 rpm at 4 °C for 15 min. The supernatant was collected, weighed, and the *WHC* was calculated using Equation (1):

$$WHC\ (\%) = \left(1 - \frac{W_1}{W_2}\right) \times 100 \quad (1)$$

W_1: weight of supernatant after centrifugation (g); W_2: fermented soymilk weight (g).

2.7. Flow Behavior and Apparent Viscosity

The flow behavior of the fermented soymilk was evaluated by a rotational rheometer (Haake Mars III, Thermo Scientific, Karlsruhe, DE) with a plate–plate measuring system (20 mm in diameter, 1 mm gap distance). Flow curves were generated by varying the shear rate from 0.001 to 300 s^{-1} over 2 min, recording the shear stress and viscosity values at the temperature of 37 °C (during the fermentation step) and 4 °C (during storage). The temperature was controlled by a heating and cooling system (Phoenix II, Thermo Scientific, Karlsruhe, Germany) combined with a Peltier system [27,28]. In addition, the apparent viscosity was obtained from flow curves at a shear rate of 50 s^{-1} at 4 °C [29]. Non-inoculated soymilk was used as the control.

2.8. Chemical Analysis

For subsequent chemical analyses, soymilk samples were frozen at −40 °C and then freeze-dried under vacuum at 15 Pa in a freeze-drier Genesis 25 ES dryer (VirTis Genesis 25ES, SP Industries Inc., Gardiner, NY, USA) for 48 h (maximum shelf temperature +20 °C). After freeze-drying, the samples were stored at room temperature. The freeze-drying treatment was necessary to guarantee the stability of the soymilk for the subsequent analytical steps. The moisture content (%) of the freeze-dried soymilk was determined according to the method described by Jung et al. [30].

2.8.1. Determination of Isoflavones

The content of isoflavones in soymilk during the fermentation stage was determined by HPLC, using a Dionex (Dionex, Sunnyvale, CA, USA) Ultimate 3000 quaternary pump equipped with a degasser, thermostated column compartment, and a diode array detector (DAD). Soymilk was sampled at 0, 3, 6, 9, and 24 h from the beginning of fermentation and subjected to freeze-drying. Then, an aliquot of 500 mg freeze-dried soymilk was subjected to a methanolic extraction, according to the method described by Fahmi et al. [31]. Briefly, 25 µL of methanolic extract was injected into a reversed-phase column (Phenomenex Luna 5 µm C18 100 Å 250 × 4.6 mm) protected with Security Guard Phenomenex (Phenomenex

Inc., Torrance, CA, USA). Elution was performed at a flow rate of 1 mL/min at 35 °C, using a solution of phosphoric acid 0.1% (eluent A) and absolute methanol (eluent B), setting the following gradient elution profile expressed as %B: 0 min 28%, 2 min 37%, 30 min 73%. Isoflavones were detected at a wavelength of 254 nm. The identification and quantification were performed using Chromeleon software ver. 6.80 through the retention times and calibration curves of daidzein, daidzin, genistein, genistin, glycitein, and glycitin, as external standards. Results were expressed as mg/100 g dry weight (D.W.).

2.8.2. Antioxidant Activity (ABTS Assay)

The 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS$^{\bullet}$+) was used for estimating the total antioxidant activity (TAA), according to the method described by Re et al. [32]. Briefly, ABTS was dissolved in water to a 7 mM concentration. ABTS radical cations (ABTS$^{\bullet}$+) were produced by reacting the ABTS methanol solution with 2.45 mM potassium persulfate. The ABTS$^{\bullet}$+ solution was diluted with cold pure methanol up to an optical density (OD) of 0.700 at 745 nm. An aliquot of 20 mg of freeze-dried soymilk sample was subjected to methanolic extraction. After 2 h, 100 µL of methanolic extract was mixed with 900 µL of the ABTS$^{\bullet}$+ solution, and the decrease in absorbance was recorded using a BioSpectrometer (Eppendorf, Hamburg, Germany). Trolox was used as standard for the calibration curve. The TAA in soymilk was assayed at 0, 3, 6, 9, and 24 h after inoculum and expressed as the Trolox equivalent antioxidant capacity (TEAC; mg Trolox Eq./100 g D.W.).

2.8.3. Thiobarbituric Acid Reactive Substance (TBARS) Assay

The TBARS assay was used to estimate lipid peroxidation using malondialdehyde (MDA) as a marker. To 40 mg of freeze-dried soymilk, 1 mL of ethanol/water solution (80:20 v/v) was added, and the suspension was vigorously shaken. Then, samples were centrifuged at 13,000 rpm, and the supernatant was used to generate the malondialdehyde–thiobarbituric acid (MDA-TBA) complex, according to the method described by Hodges et al. [33], with some modifications. Absorbances were detected at 532, 440, and 600 nm, using a BioSpectrometer (Eppendorf, Hamburg, Germany). An aqueous solution of malondialdehyde bis(dimethyl acetal) was used as a standard for the calibration curve. The quantities of MDA during the fermentation phase were calculated using the modified equation reported by Landi [34].

2.9. Statistical Analysis

Statistical analysis was performed by analysis of variance (ANOVA), followed by Tukey's multiple comparisons, while a *t*-test was used for the analysis of the significance of the apparent viscosity during the fermentation stage, using SPSS Statistics 21 software (IBM Corp., Armonk, NY, USA). The calculations were performed on data obtained from the analysis carried out on three independent aliquots (n = 3) obtained from a single initial mass of soymilk after starter microbial inoculation. All data were expressed as a mean ± standard deviation (±SD).

3. Results

3.1. Preliminary Fermentation Trial

Although the fermentation kinetics have a similar pattern, preliminary tests revealed, as shown in Figure 1 and Tables S1 and S2 (Supplementary Materials), that 37 °C was the optimal temperature for the fermentation of soymilk using *Lp. plantarum* LP95. In detail, at this temperature, the viable cell counts increased progressively from 7.32 log CFU/mL to a value greater than 9 log CFU/mL, after 9 h of fermentation. Moreover, as shown in Figure 1, after 9 h at 37 °C, the fermented soymilk reached values below pH 5.0, lower than those recorded in tests at 20 °C (pH 5.98) and 28 °C (pH 5.31).

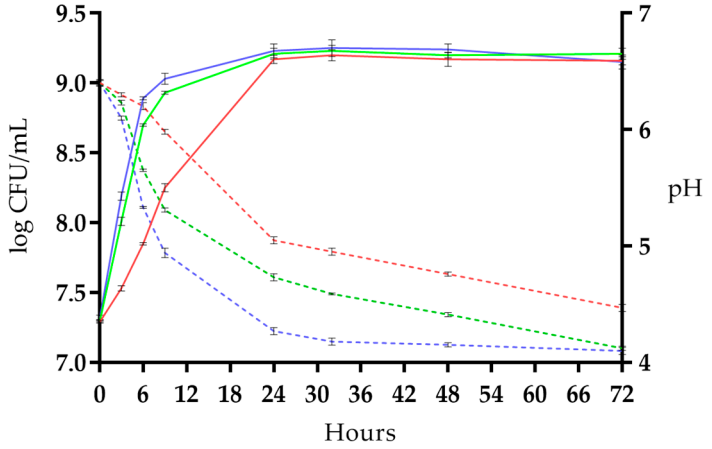

Figure 1. Viable cell count (log CFU/mL) of *Lp. plantarum* LP95 (continuous line) and pH values (dashed line) during the fermentation of soymilk at 20 °C (red line), 28 °C (green line), and 37 °C (blue line). Error bars indicate the standard deviation of the means (n = 3).

3.2. Microbiological and Physicochemical Parameters during Fermentation and Storage

Figure 2 shows the viable cell count and pH values using *Lp. plantarum* LP95 in soymilk during the fermentation and storage stages (see also Table S3). As inferred from the viable cell count of *Lp. plantarum* LP95, it reached a value of approximately 9 log CFU/mL during the fermentation phase, which remained constant over the following seven days. After 7 days, a fast decrease in viable cell count occurred, followed by a slower decrease, starting on the 28th day. However, the viable cell count remained above 7 log CFU/mL for up to 49 days of storage. Regarding the pH value of fermented soymilk, an acidification process was evident during fermentation (from 6.5 to 4.2), while during storage, the pH value remained unchanged. In addition, the WHC of fermented soymilk was determined after 24 h, and it reached a value of 43.1 ± 0.3%.

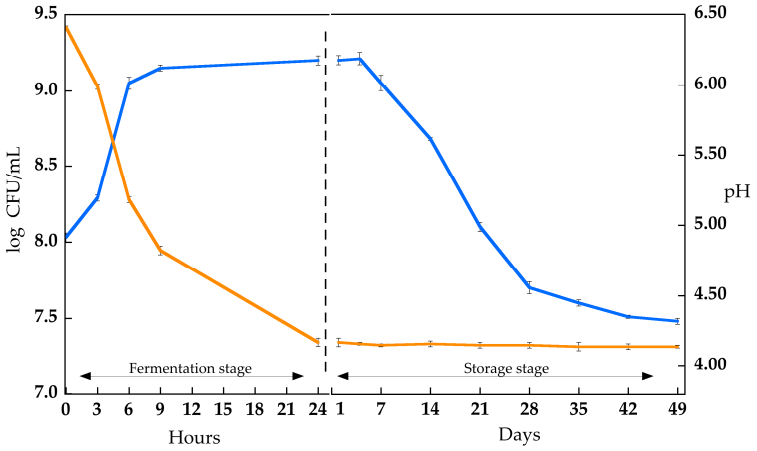

Figure 2. Viable cell count (log CFU/mL) of *Lp. plantarum* LP95 (blue line) and pH values (orange line) during soymilk fermentation (37 °C; 24 h) and fermented soymilk storage (4 °C; 49 days). Error bars indicate the standard deviation of the means (n = 3).

3.3. Flow Behavior and Apparent Viscosity of Fermented Soymilk

It is well-known that the correlation between the shear stress (τ) and the shear rate ($\dot{\gamma}$) defines the flow behavior of a sample, which can be classified as Newtonian behavior if the viscosity (η) does not vary with deformation or non-Newtonian behavior when the viscosity varies as a function of the applied shear rate or shear stress. In this study, the apparent viscosity of fermented soymilk was measured to give preliminary rheological information concerning fermented soymilk. The flow behavior of fermented soymilk during both

fermentation (37 °C) and storage (4 °C) stages was evaluated, and the obtained curves are depicted in Figure 3A,B, respectively. In detail, all samples showed a non-Newtonian shear-thinning behavior, so the apparent viscosity decreased when the shear rate increased. In Figure 3A, it is possible to observe how fermentation led to changes in the soymilk, with a visible increase in viscosity after 24 h of fermentation. During the storage stage (Figure 3B), a slight decrease in the viscosity of the sample was observed, starting after 21 days. The consistency coefficient (K) and flow behavior index (n) were obtained as described by Hamet et al. [35], fitting the flow curves with the Ostwald–de Waele model, and the results are reported in Table S4. Moreover, the apparent viscosities of the fermented soymilk at a shear rate of 50 s^{-1} during the fermentation and storage stages are shown in Figure 4A,B, respectively, while the numerical data are reported in Tables S5 and S6. In detail, after 24 h of fermentation, the values of apparent viscosity were different compared to the sample at 0 h. However, after clot rupture at 7 days of storage (4 °C), the highest level of apparent viscosity occurred, while at 21 days, a rapid decline was observed.

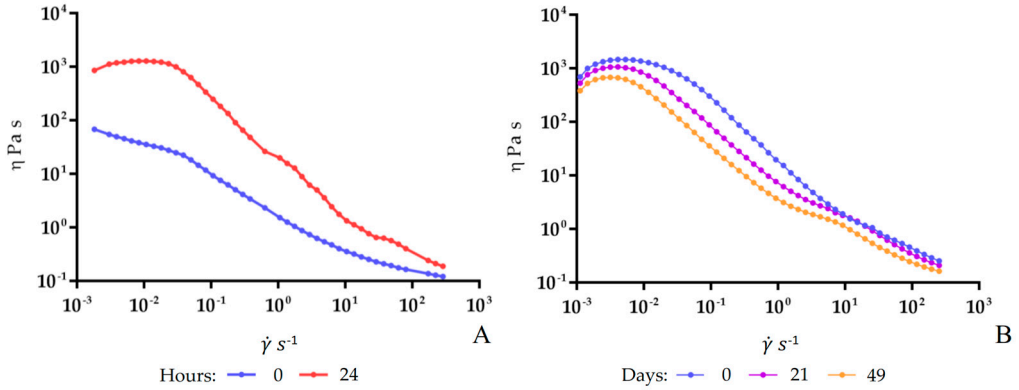

Figure 3. Viscosity curves of soymilk during fermentation using *Lp. plantarum* LP95 as a starter at 37 °C (**A**) and fermented soymilk during storage at 4 °C (**B**) at different times: viscosity (η) as a function of the shear rate ($\dot{\gamma}$).

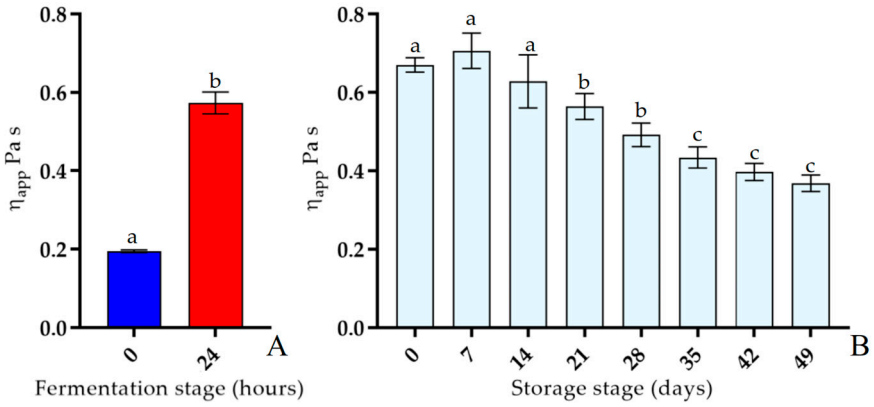

Figure 4. Apparent viscosity at 50 s^{-1} of soymilk during fermentation using *Lp. plantarum* LP95 as a starter at 37 °C (**A**) and fermented soymilk during storage at 4 °C (**B**) at different times. All values are expressed as the mean ± standard deviation (n = 3). Different lowercase letters in each bar indicate significant differences ($p < 0.05$).

3.4. Bioconversion of Isoflavones during Fermentation

Soymilk fermentation, using *Lp. plantarum* LP95 as a starter, significantly increased isoflavones in the aglycone-form. In detail, the results reported in Table 1 showed that the glycosylated isoflavones in soymilk, immediately after the inoculum (0 h), contained daidzin, glycitin, and genistin (11.26 ± 0.27, 39.34 ± 1.00, 40.39 ± 1.27 µmol/100 g D.W.), while the aglycone derivatives were daidzein, glycitein, and genistein (25.64 ± 0.02,

1.58 ± 0.18, and 21.58 ± 0.39 µmol/100 g D.W.). The content of daidzein, glycitein, and genistein increased after 24 h of fermentation, reaching the highest levels of 48.45 ± 1.30, 5.10 ± 0.16, and 56.35 ± 1.02 µmol/100 g D.W., respectively. The moisture content (%) of freeze-dried soymilk was 0.912 ± 0.008.

Table 1. Deglycosylation of soymilk isoflavones into aglycones during the fermentation stage of soymilk using *Lp. plantarum* LP95 as a starter. All values are expressed as the mean ± standard deviation (n = 3).

Isoflavones (µmol/100 g D.W.)	Fermentation Stage (hours)				
	0	3	6	9	24
Daidzin	11.26 ± 0.27 ^{a}	11.08 ± 0.26 ^{a}	0.57 ± 0.02 ^{b}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}
Glycitin	39.34 ± 1.00 ^{a}	37.78 ± 0.55 ^{b}	1.45 ± 0.10 ^{c}	0.00 ± 0.00 ^{d}	0.00 ± 0.00 ^{d}
Genistin	40.39 ± 1.27 ^{a}	40.11 ± 0.02 ^{a}	4.60 ± 0.09 ^{b}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}
Daidzein	25.64 ± 0.02 ^{b}	25.76 ± 1.11 ^{b}	47.71 ± 0.72 ^{a}	48.45 ±1.74 ^{a}	48.45 ± 1.30 ^{a}
Glycitein	1.58 ± 0.18 ^{d}	1.65 ± 0.04 ^{d}	2.61 ± 0.17 ^{c}	3.69 ± 0.01 ^{b}	5.10 ± 0.16 ^{a}
Genistein	21.58 ± 0.39 ^{c}	21.77 ± 0.80 ^{c}	51.14 ± 0.84 ^{b}	54.75 ± 2.11 ^{a}	56.35 ± 1.02 ^{a}

Different lowercase letters (a–d) in each row indicate significant differences ($p < 0.05$).

3.5. Changes in Antioxidant Activity during Fermentation

During the fermentation stage, the measured values of TAA were significantly different compared to the soymilk immediately after inoculum (0 h). In particular, the results illustrated in Figure 5A and the numerical data reported in Table S7 have shown that the TEAC at 0, 3, 6, 9, and 24 h was 123.35 ± 1.58, 110.30 ± 6.03, 121.42 ± 5.26, 105.79 ± 2.34, and 108.76 ± 3.25 mg Trolox Eq./100 g D.W., respectively. Specifically, the percentage decrease in TEAC was already about 10% at 3 h; at 6 h, it was only 2%, and at 9 and 24 h, it was around 13% compared to the sample at the beginning of fermentation.

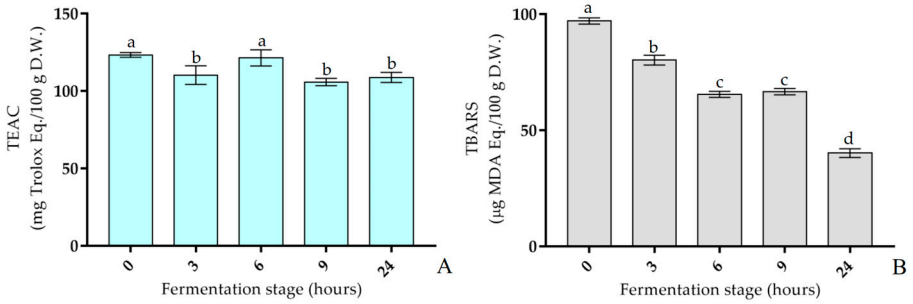

Figure 5. Variations in TEAC expressed as mg Trolox Eq./100 g D.W. (**A**) and TBARS expressed as µg MDA Eq./100 g D.W. (**B**) in soymilk during fermentation using *Lp. plantarum* LP95 as a starter. All values are expressed as the mean ± standard deviation (n = 3). Different lowercase letters in each bar indicate significant differences ($p < 0.05$).

3.6. Variation in MDA during Fermentation

The evolution of MDA levels during fermentation was assayed. In particular, the detected values showed significant differences during the fermentation stage. As reported in Figure 5B and Table S8, the highest level of MDA concentration was detected at 0 h (97.11 ± 1.33 µg MDA Eq./100 g D.W.); however, it significantly decreased at 3 h (80.26 ± 2.12 µg MDA Eq./100 g D.W.), while between 6 and 9 h (65.53 ± 1.30; 66.68 ± 1.35 µg MDA Eq./100 g D.W., respectively), there were no significant differences between the detected values. Finally, at 24 h, the MDA value reached its lowest level (40.17 ± 1.88 µg MDA Eq./100 g D.W.).

4. Discussion

The formulation of functional foods based on the use of fermentative starters that also possess probiotic properties is becoming an aspect of growing interest in the field of food biotechnology. In this investigation, *Lp. plantarum* LP95 showed good fermentation

performance and some properties that overall improved the biofunctional characteristics of unfermented soymilk. It was also inferred that carbohydrates naturally present in soymilk, such as stachyose, raffinose, sucrose, glucose, and fructose, act as substrates for fermentation by LAB, leading to a lowering of pH due to the release of lactic acid [36,37]. The used starter LP95, after 24 h of fermentation at 37 °C, produced a compact clot. After clop rupture, the determined WHC was similar to data obtained by other authors using different LAB strains in soybean-based products [38–40].

The observed viscosity changes that occurred during fermentation were also attributable to protein coagulation phenomena [41]. In fact, as described by Xu et al. [42], the protease activity of *Lp. plantarum* might partially hydrolyze soy proteins by lowering soymilk pH. This negatively charges the denatured soy protein groups, leading to progressive coagulation. In addition, the ability to produce EPSs from *Lp. plantarum* LP95 [25] could have a positive influence on the coagulation phase [5]. To clarify this aspect, comparative studies will be conducted in the future on soymilk fermented by *Lp. plantarum* LP95 using non-EPS-producing LAB strains or coagulation with calcium chloride [43,44] as references, with and without the addition of EPSs [45].

The study of the apparent viscosity of samples is important to evaluate how fermentation could affect the sensorial quality of the final product. In general, the properties of fermented products are influenced by different factors, such as the type of microorganisms used and the duration and temperature at which fermentation takes place [46].

Samples analyzed in this study possessed a non-Newtonian shear-thinning behavior, where the apparent viscosity decreased when the shear rate increased. This behavior was also observed for other vegetable-fermented types of milk [35,39,47]. The flow behavior of the fermented product was then followed during the refrigerated storage phase. The same behavior observed during fermentation was also found during storage for up to the 14th day, with a drop in viscosity values in the following days. The physicochemical changes could also affect the texture and mouthfeel of the final product, so the apparent viscosity at a shear rate of 50 s^{-1} was obtained, as it can be related to the oral perception during the consumption of semisolid products [48,49].

The ability of this *Lactobacillus* to increase the viscosity of soymilk could also be related to the probable presence of EPSs, in agreement with previous studies [35,50–52]. In this respect, we can report that during the storage of samples containing fermented soymilk stored at 4 °C, the apparent viscosity remained substantially constant.

The bioavailability of glycosidic isoflavones first requires enzymatic hydrolysis of the sugar moiety, which occurs in the microvillous zone of enterocytes in the intestine [53]. Several LAB strains show specific β-glucosidase enzyme activity that hydrolyzes isoflavone glycosides to aglycones [54]. In detail, the fermentation of soymilk using *Lp. plantarum* LP95 as a starter culture confirmed the complete biotransformation of isoflavone glucosides into their aglyconic forms, which occurred after 6 h during the fermentation. Vegetable polyphenols introduced into a diet with soymilk act as an exogenous antioxidant system. In fact, they have an important antioxidant activity against the negative effects of free radicals, preventing oxidative stress and cell damage [55]. Data obtained on the total antioxidant capacity of the fermented soymilk samples were determined by the ABTS radical scavenging. In the first 3 h of the fermentation process, there was a slight reduction in the antioxidant activity, probably due to a depletion of antioxidant molecules by the bacterial cells used to mitigate oxidative stress [20]. After six hours, there was an increase in the antioxidant activity of the fermented soymilk, probably attributable to the deglycosylation by bacterial bioconversion of isoflavone glycosides. After, the antioxidant activity returns to a decrease, probably due to the scavenging of isoflavonoids [56] produced during bacterial metabolism.

Furthermore, an important parameter to monitor during food processing is the oxidative state of the final product. The oxidation of lipids, which has been extensively studied, proceeds via a free-radical chain mechanism, giving rise to numerous by-products. The main one of these is MDA, which is, in fact, a marker of oxidative damage in biological

systems [57]. Monitoring lipid oxidation is important, as this process leads to the loss of nutritional factors and the development of toxic compounds in food, causing various diseases, such as atherosclerosis [58]. The detection of TBARS is frequently used to determine lipid oxidation. However, as reported by Ghani et al. [59], this measure does not indicate the antioxidant mechanism of action. Despite this potential limitation, there are features of the TBARS test that make it useful as a complement to other widely used screening tests, such as the TAA. In our study, the TBARS assay showed a progressive reduction in MDA levels during the fermentation of soymilk using *Lp. plantarum* LP95. Additionally, this trend coincides with the increase in the amount of detected isoflavone aglycones, and this may suggest a correlation between the two events.

5. Conclusions

In this article, we presented a study on the *Lp. plantarum* LP95 strain, which has good potential for improving the quality of fermented soymilk to develop functional food products. The enhancement of the antioxidant activity of soymilk has been shown.

The increase in isoflavone aglycone content during fermentation was a result of β-glucosidase activity toward isoflavone glucosides. This process allowed the obtaining of a soy beverage with enhanced antioxidant capacity able to contribute to the improvement in the health and nutritional status of consumers.

Furthermore, we also report that without using chemical additives, the soymilk obtained and stored at 4 °C has a good shelf life of up to 14 days.

Based on this evidence, *Lp. plantarum* LP95 appears to be a promising candidate as a starter culture to obtain a fermented soymilk rich in isoflavone aglycones and with enhanced health-promoting properties.

To validate this application, future investigations will be conducted on the shelf life and on the organoleptic and nutritional qualities of soymilk fermented by this bacterium.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antiox12071442/s1, Table S1: Viable cell count (log CFU/mL) of *Lp. plantarum* LP95 during the preliminary fermentation trial of soymilk at 20 °C, 28 °C, and 37 °C, Table S2: Changes in pH during the preliminary fermentation trial of soymilk with *Lp. plantarum* LP95 at 20 °C, 28 °C, and 37 °C, Table S3: Changes in viable cell count (log CFU/mL) and pH in soymilk during the fermentation (37 °C) and storage stage (4 °C) using *Lp. plantarum* LP95 as a starter, Table S4: Values of consistency coefficient (*K*) and flow behavior index (*n*) obtained by fitting the flow curves with the Ostwald–de Waele model, Table S5: Apparent viscosity (Pa s) at 50 s^{-1} of soymilk during the fermentation stage at 37 °C using *Lp. plantarum* LP95 as a starter, Table S6: Apparent viscosity (Pa s) at 50 s^{-1} of fermented soymilk during the storage stage at 4 °C using *Lp. plantarum* LP95 as a starter, Table S7: Variations in total antioxidant activity (TEAC; mg Trolox Eq. 100 g^{-1} D.W.) in soymilk during the fermentation stage using *Lp. plantarum* LP95 as a starter, Table S8: Variations in thiobarbituric acid reactive substance (TBARS; μg MDA Eq. 100 g^{-1} D.W.) in soymilk during the fermentation stage using *Lp. plantarum* LP95 as a starter.

Author Contributions: Conceptualization, C.D.M. and M.I.; methodology, C.D.M., M.I., M.C., F.L. (Francesco Lopez), G.P., A.F., and B.T.; software, M.C. and F.L. (Francesco Letizia); validation, G.A., F.L. (Francesco Lopez), and M.C.; formal analysis, F.L. (Francesco Letizia), M.C., C.D.M., F.L. (Francesco Lopez), G.P., B.T., and A.F.; writing—original draft preparation, F.L. (Francesco Letizia), C.D.M., and M.I.; writing—review and editing; F.L. (Francesco Letizia), M.I., A.F., G.P., C.D.M., and M.C.; project administration, C.D.M. and M.I. All authors have read and agreed to the published version of the manuscript.

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Application of *Lactiplantibacillus plantarum* LP95 as a Functional Starter Culture in Fermented Tofu Production

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Abstract: Several studies have shown that lactic acid bacteria (LAB) fermentation plays an important role in the development and application of soy-based products and could increase their nutritional values and content of bioactive substances. *Lactiplantibacillus plantarum* LP95 has shown in previous studies to be a promising candidate as a probiotic and microbial culture in fermented soymilk production. In this study, the suitability of *Lp. plantarum* LP95 as a functional starter culture in tofu production was verified, with a focus on evaluating the isoflavone and amino acid content in the final product after 21 days of storage at 4 °C. *Lp. plantarum* LP95 was found able to ferment monosaccharides and disaccharides naturally present in soymilk (D-glucose, D-fructose, D-galactose and D-sucrose) after 24 h while leaving the content of galacto-oligosaccharides (stachyose and raffinose) unaffected. The rich amino acid profile of tofu has undergone some quantitative but not qualitative variations compared to the soy milk used, highlighting the high nutritional value of the product obtained. The enzymatic activity of *Lp. plantarum* LP95 allowed the release of isoflavone aglycones (daidzein, glycitein and genistein) that were not further metabolized during the fermentation phase of soymilk and during storage at 4 °C. In addition, *Lp. plantarum* LP95 showed a good viability after 21 days of tofu storage at 4 °C. The results obtained highlighted the suitability of this LAB strain to be used as a microbial culture capable of playing a pro-technological role in the production of fermented tofu, which has good nutritional and functional properties.

Keywords: fermented soy product; *Lactiplantibacillus plantarum*; tofu

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1. Introduction

Tofu, a very popular food in Eastern Asia, can be produced from soymilk, through a coagulation process. Also known as bean curd, it is a product with high nutritional value due to its high protein content and a low saturated fat content. This soybean product has become increasingly popular due of its inclusion in hypocaloric, vegetarian and vegan diets [1]. Tofu is categorized by texture, or consistency determined by water content and it is mainly produced and marketed as Extra Firm, Firm, Soft, and Silken Tofu [1,2].

The basic method of making tofu is the same and include three fundamental steps, resulting in specific characteristics of the product type: (i) soymilk extraction and solid content, (ii) coagulation method (types of coagulants, breaking or not after curd formation), and (iii) pressing or not [3]. During tofu production, soymilk is coagulated by adding acids, salts, and enzymes. The acid coagulation methods cause isoelectric precipitation and thus gelation of soy proteins, while enzyme coagulants (e.g., transglutaminase) can cause isopeptide bonds among the ξ-amino group in the lysine residue and the amine group of glutamine residue. Finally, with salts (e.g., calcium sulphate, calcium chloride, calcium

acetate, calcium lactate, and magnesium chloride), the formation of a three-dimensional network structure occurs because of salt bridges cross-connecting proteins [4].

Apart from conventional screening procedures for the evaluation of probiotics for human use, industries have stimulated research to implement further selective criteria for the identification of bacteria with particular characteristics to be used as microbial cultures or dietary supplements in the production of functional foods.

For a long time, lactic acid bacteria (LAB) have been used in fermented foods and beverages as starter cultures, due to their capacity to improve their organoleptic, nutritional, technological and preservation characteristics [5].

Soymilk is a suitable habitat for these microorganisms since they rapidly grow in this medium, as already reported by different authors [6–9]. In China, mainly in rural areas fermented soybean whey, rich in LAB, has been used as a traditional tofu coagulant for more than 600 years [10]. Fermented soybean products contain a microbial community, with LAB as predominant microorganisms, that play an important fermentative role and therefore influence their sensory and chemical-physical characteristics [11].

LAB fermentation can improve the nutritional values of soybean-derived products causing the conversion glycosylated and/or acylated isoflavones to aglycone, consequently favoring their absorption [12]. In addition, fermentation of soymilk by selected LAB strains and the following precipitation of proteins has been demonstrated to prevent or delay undesired microbial and chemical spoilage, increasing the quality and the stability of the end product [13].

Among LAB species, *Lactiplantibacillus plantarum* has been widely reported as a microbial food culture [14–17] including its use as a starter in the production of fermented tofu [10].

Based on previous studies, *Lp. plantarum* LP95 has demonstrated the ability to inhibit the growth of certain human opportunistic pathogens, possess β-glucosidase, and synthesize exopolysaccharide (EPS) and γ-aminobutyric acid (GABA). Moreover, enhance the antioxidant activity of fermented soymilk [18,19]. The aim of this research was to evaluate some compositive characteristic of soft tofu-type product obtained through fermentation with *Lp. plantarum* LP95 mainly focusing on the sugar, isoflavone and amino acid content.

2. Materials and Methods

2.1. Bacterial Strain

In this study, *Lp. plantarum* LP95 (Genbank accession number: OM033654), isolated from fermented pollen (bee bread), was cultured at 37 °C in 500 mL of MRS medium (de Man, Rogosa and Sharpe Broth, Oxoid Ltd., Basingstoke, Hampshire, UK). After 12 h, bacterial cells were harvested by centrifugation (10,000 rpm, 5 min, 4 °C) and washed with sterile saline solution (0.9% *w*/*v* NaCl). The supernatant was discarded, the cell pellet was recovered and inoculated in soymilk. The preliminary estimation of the cell concentration in MRS was carried by microscopy using a Thoma counting chamber. Subsequently, viable count was enumerated after inoculation and during tofu production, in MRS agar plates after incubation at 37 °C for 48 h in anaerobically condition.

2.2. Production of Fermented Soymilk and Tofu

The soymilk obtained from 1600 g of yellow soybeans was prepared as described by Letizia et al. [19]. Figure 1 shows a flowchart for the production of fermented tofu. Briefly, each 400 g of mature yellow organic soybeans (*Glycine max*, purchased from BV&FdL S.r.l.; Bentivoglio, BO, Italy) was soaked in 1200 mL sterile distilled water for 12 h [20]. Subsequently, soybeans were drained, dried blended by adding 1400 mL of water and sterilized. Finally, 4500 mL of soymilk was shaken and inoculated with *Lp. plantarum* LP95 (10^8 CFU/mL) and divided into 3 aliquots.

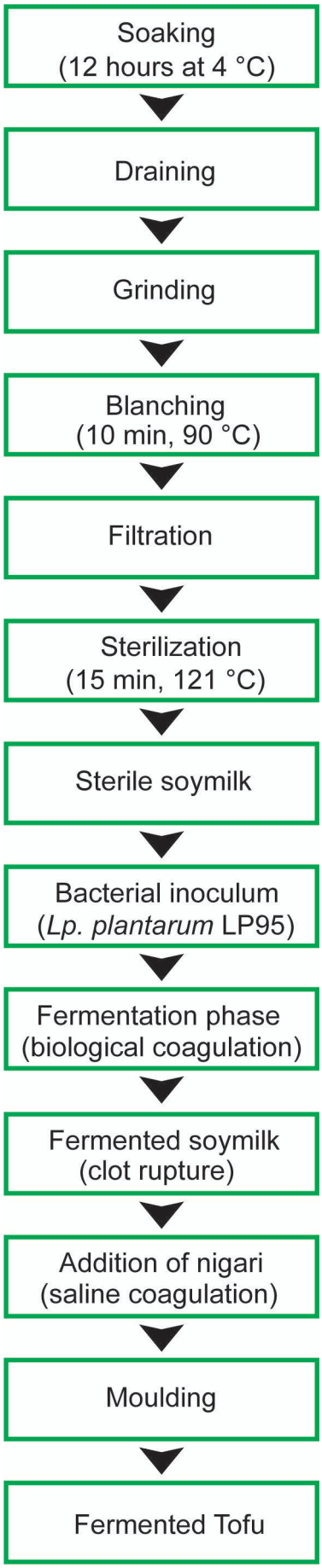

Figure 1. Flow chart of fermented tofu production.

After 24 h at 37 °C, the clot was broken and nigari 3% *w*/*v* (marketed by La finestra sul cielo, by Natura S.r.l., Villareggia, Italy) was added to promote the coagulation. After 1 h, the fermented soymilk was poured into disposable plastic molds for the draining of whey and finally stored in a sealed sterile bag at 4 °C.

2.3. Physical Parameters

Spontaneous syneresis of tofu was made following the method described by Amatayakul et al., with some modifications [21]. Briefly, after 24 h of 4 °C storage the exuded liquid was measured and the equation (1) was used to calculate the syneresis [22]. The method described by Jung et al. [23], was followed to determine moisture content (%) of the freeze-dried soymilk, while a water activity meter (AquaLab CX-2; Pullman, WA, USA) was used to measure water activity (a_w) of freeze-dried samples at 25 ± 1 °C [24]. The pH value was estimated using Hanna Edge HI2020 pH meter equipped with food probe (Hanna Instruments, Woonsocket, RI, USA).

$$Syneresis(\%) = \frac{W_i - W_f}{W_i - W_c} \times 100 \quad (1)$$

where W_i is the initial weight (g) of the plastic molds with tofu, W_f is the final weight (g) of plastic molds with tofu after whey removal and W_c is the weight (g) of the plastic molds.

2.4. Chemical Analysis

To perform the chemical evaluations, unfermented soymilk, fermented soymilk (after 24 h of fermentation) and tofu (after storage of 21 days at 4 °C) were frozen at −40 °C and then freeze-dried under vacuum at 15 Pa by using a freeze-drier Genesis 25 ES dryer (VirTis Genesis 25ES; SP Industries Inc., Gardiner, NY, USA), for 48 h (maximum shelve temperature +20 °C). Freeze-dried samples were stored at room temperature.

2.4.1. Sugar Metabolism

Sugars content was determined on an aliquot of 500 mg of fresh soymilk at 0, 3, 6, 9, 12, 18 and 24 h of fermentation. Samples were properly diluted (1:10–1:40) in ultrapure water, centrifugated at 14,000 rpm for 10 min at 4 °C and supernatants were filtered through a 0.44 µm nylon syringe filter (Merck Millipore, Burlington, MA, USA) and stored at 4 °C for subsequently analytical steps. Sugar separation and detection were performed via High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD), using a Dionex system ICS6000 (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 20 µL of sample were injected into a Dionex Carbopac PA100 analytical column (250 × 2 mm) (Thermo Fisher Scientific, Waltham, MA, USA) and eluted at a flow rate of 0.25 mL/min, at 25 °C, using eluent A (water) and eluent B (100 mM NaOH), following the gradient elution profile: %B: 0 min 17%, 8 min 17%, 35 min 100%, 45 min 100%, 45.1 min 83%, 55.0 min 83%. The waveform time/potential expressed as second/Volt were: 0.00/0.10, 0.20/0.10, 0.40/0.10, 0.41/−2.00, 0.42/−2.00, 0.43/0.60, 0.44/−0.10, 0.50/−0.10. The electrochemical cell consisted of a 1 mm diameter gold working electrode, and an Ag/AgCl reference electrode. A Chromeleon 7 (Thermo Fisher Scientific, Waltham, MA, USA) was used for the instrument control, data collection and total quantification of sugars, comparing the retention times and internal peak areas of reference standards.

2.4.2. Determination of Isoflavones

An HPLC Dionex (Sunnyvale, CA, USA) Ultimate 3000 binary pump was used to determine the content of isoflavones in the soymilk samples, together with a column compartment thermostated at 35 °C, and a Diode Array Detector (DAD). An aliquot of 500 mg freeze-dried soymilk was extracted with methanol (1 mL), following the method of Fahmi et al. [25]. Briefly, 25 µL of methanolic extract was injected into a reversed-phase column (Phenomenex Luna 5 µm C18 100 Å, 250 × 4.6 mm) protected with a Phenomenex Security Guard (Torrance, CA, USA). Compounds were eluted at a flow

rate of 1 mL/min, at 35 °C, using, as eluent A, 0.1% phosphoric acid and, as eluent B, absolute methanol. In detail the linear elution profile was: %B: 0 min 28%, 2 min 37%, 30 min 73%. Isoflavones were detected at a wavelength of 254 nm. The identification and quantification were made using the Chromeleon software ver. 6.80, through retention times and calibration curves of daidzein, daidzin, genistein, genistin, glycitein, and glycitin, as references external standards.

2.4.3. Amino Acids Profile

The primary L-amino acid content of the freeze-dried samples (20 mg) was determined after ethanol/water (40/60 *v*/*v*; 1 mL) extraction. Subsequently, samples were centrifugated at 14,000 rpm for 5 min, at 4 °C, and supernatants were filtered through a 0.44 µm nylon syringe filter (Merck Millipore, Burlington, MA, USA) and used for amino acid derivatizations and quantification through a Prominence/Nexera X2 HPLC system (Shimadzu, Kyoto, Japan), equipped with Degasser DGU-20A5R, Binary Pump LC-30AD, Autosampler SIL-30AC, Column Oven CTO-20A, DAD Detector SPD-M20A and Fluorescence Detector RF-20Axs. Briefly, for derivatization, 20 µL of extract were mixed with 40 µL of *o*-Phthalaldehyde (OPA) reagent (Agilent, Santa Clara, CA, USA). Finally, 30 µL of OPA-derivatives were injected into a reverse-phase column (ZORBAX Eclipse Plus C18, 4.6 × 250 mm 5 µm; Agilent, Santa Clara, CA, USA), protected with a SecurityGuard Phenomenex (Torrance, CA, USA). The elution was performed at a flow rate of 0.85 mL/min, at 27 °C, using an eluent A (50 mM sodium acetate/0.3% tetrahydrofuran/20%methanol, pH 6.4) and an eluent B (100% methanol) [26]. OPA derivatives were spectrofluorimetric detected at 340 nm excitation and 450 nm emission wavelengths. The identification and quantification of amino acids was carried out using a LabSolutions LCGC v. 5.90 software (Shimadzu, Kyoto, Japan), comparing the retention times and internal peak areas to reference standards. On the hydroalcoholic extracts obtained as illustrated above, the quantification of proline was conducted using the ninhydrin-based method [27].

2.4.4. ABTS Antioxidant Activity

The Total Antioxidant Activity (TAA) in samples was estimated by means of the 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$), by decolorization of the 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$), generated in organic phase, following the method described by Re et al. [28]. Briefly, ABTS was dissolved in methanol to a 7 mM concentration. ABTS radical cations ($ABTS^{\bullet+}$) were produced by reacting the ABTS methanol solution with 2.45 mM potassium persulfate. The $ABTS^{\bullet+}$ solution was diluted with cold pure methanol up to an optical density (OD) of 0.700 at 745 nm. An aliquot of 40 mg of freeze-dried soymilk sample was extracted with 1 mL of methanol. After 2 h, 900 µL of the ABTS•+ solution were added to 100 µL of methanolic extract and the decrease of absorbance was recorded through a BioSpectrometer (Eppendorf, Hamburg, Germany) [19]. Trolox was used as a standard for the calibration curve and the TAA was expressed as Trolox Equivalent Antioxidant Capacity (TEAC).

2.4.5. DPPH Antioxidant Activity

The DPPH assay measured the TAA decolorizations of the 2,2-Diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical generated in organic phase, using the method described by Rahman et al., with some modifications [29]. Briefly, an aliquot of 40 mg of freeze-dried samples were subjected to an extraction with methanol (1 mL). After 2 h, 100 µL of methanolic extracts were mixed with 900 µL of the 70 µM $DPPH^{\bullet}$ methanol solution and the decrease of absorbance at 515 nm was recorded after 15 min, using a BioSpectrometer (Eppendorf, Hamburg, Germany). Trolox was used as a standard for the calibration curve and the TAA was expressed as Trolox Equivalent Antioxidant Capacity (TEAC).

2.4.6. Total Phenolics

The Folin–Ciocalteu method was used to determine the total phenolic content (TPC) [30]. In details, 40 mg of freeze-dried tofu was extracted with methanol (1 mL) and then stored for 2 h at 4 °C. Finally, 50 µL of the Folin–Ciocalteu reagent (1:5 *v*/*v*) was added to 50 µL of the extract; they were mixed and the reaction was stopped with 100 µL of 350 mM sodium hydroxide solution. Absorbance was recorded using a BioSpectrometer (Eppendorf, Hamburg, Germany), at 760 nm. Gallic acid monohydrate (GA) was used as a standard for the calibration curve.

2.5. Statistical Analysis

Statistical analysis was performed by the analysis of variance (ANOVA), followed by Tuckey's multiple comparisons using SPSS Statistics 21 software (IBM Corp., Armonk, NY, USA). Data analysis was performed from three independent replicate (n = 3) and all data were expressed as a mean ± standard deviation (±SD).

3. Results

3.1. Fermentation Kinetics

The viability of *Lp. plantarum* LP95 was monitored during fermentation and tofu production until the 21th day of storage (Table 1). After 24-h fermentation the viable cell count increased from about 8.0 to 9.5 log CFU/mL, while during the tofu storage phase at 4 °C, there was a decrease in the viable cells, reaching 7.7 log CFU/g after 7 days and 6.0 log CFU/g after 21 days. As for the pH value, as reported in Table S1 (Supplementary Materials), fermentation caused the pH to drop from 6.4 to pH 4.1 in fermented soymilk, reaching a value of 3.82 in tofu after 21 days.

Table 1. Viable cell counts of *Lp. plantarum* LP95 during fermentation of soymilk (log CFU/mL) at 37 °C and of tofu storage (log CFU/g) at 4 °C. All values are expressed as mean ± standard deviation (n = 3).

	Days of Soymilk Fermentation		**Days of Tofu Storage**		
	0	1	7	14	21
Viable cell count	8.07 ± 0.06 ^{b}	9.52 ± 0.11 ^{a}	7.76 ± 0.29 ^{b}	6.00 ± 0.50 ^{c}	5.83 ± 0.23 ^{c}

Different lowercase letters (a–c) in row indicate significant differences ($p < 0.05$).

3.2. Physical Parameters

The syneresis value of tofu after 24 h was 22%. The moisture content in the freeze-dried unfermented, fermented soymilk and tofu samples was 3.30 ± 0.10%, 3.73 ± 0.49% and 4.1 ± 0.93% respectively, while the a_w of the same samples was 0.26 ± 0.05, 0.21 ± 0.02 and 0.16 ± 0.01, respectively.

3.3. Quantitative Changes in Sugars during the Fermentation of Soymilk

The ability of LAB to ferment soymilk sugars is related to a specific enzymatic activity. In particular, quantification of the soymilk monosaccharides (D-glucose, D-fructose and D-galactose), disaccharide (D-sucrose), and oligosaccharides (D-raffinose and D-stachyose), was performed at 0, 3, 6, 9, 12, 18 and 24 h of fermentation after *Lp. plantarum* LP95 inoculum. In detail, the fermentation curves of soymilk carbohydrate are showed in Figure 2 and numerical data are reported in Table S2. Results highlighted that glucose, fructose, galactose and sucrose were totally metabolized during 24-h fermentation. Specifically, after 3 h, glucose, fructose and sucrose contents decreased of about 65%, 43% and 11% respectively, continuing to decline of about 80%, 62% and 30%, respectively, after 6 h. The galactose content remained unchanged for the first 3 h, showing a rapid 86.0% loss after 6 h. From 9 to 24 h a slower reduction of sugar catabolism was detected, with more than 90% of monosaccharides completely fermented at the end. About 50% of sucrose remained

unfermented at 12 h and about 7% at 24 h. The content of raffinose and stachyose remained unchanged during 24 h of fermentation.

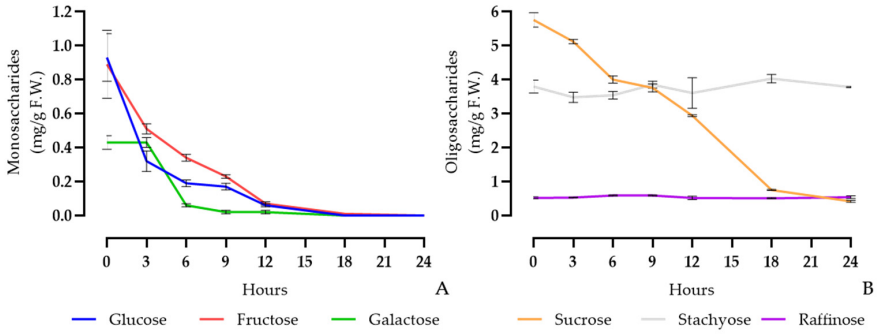

Figure 2. Quantitative changes in sugars during fermentation of soymilk using *Lp. plantarum* LP95 as starter: glucose, fructose, galactose (**A**); sucrose, stachyose and raffinose (**B**). Error bars indicate the standard deviation of the mean (n = 3).

3.4. Bioconversion of Isoflavones

Soymilk fermentation, using *Lp. plantarum* LP95 as a starter, caused a significant increase in the conversion of isoflavones into the aglycone-forms. In detail, as reported in Table S3, the glycosylated isoflavones in unfermented soymilk were daidzin, glycitin, and genistin (0.11 ± 0.01, 0.27 ± 0.05, 0.30 ± 0.01 µmol/g on dry weight; D.W., respectively), while their aglycosilated forms were daidzein, glycitein, and genistein (0.25 ± 0.03, 0.03 ± 0.01, 0.21 ± 0.03 µmol/g D.W., respectively). As showed in Figure 3, the content of daidzein, glycitein, and genistein increased in fermented soymilk (after 24 h), while they remained unchanged in tofu (after 21 days), reaching the highest levels of 0.50 ± 0.01, 0.094 ± 0.01, and 0.50 ± 0.01 µmol/g D.W., respectively.

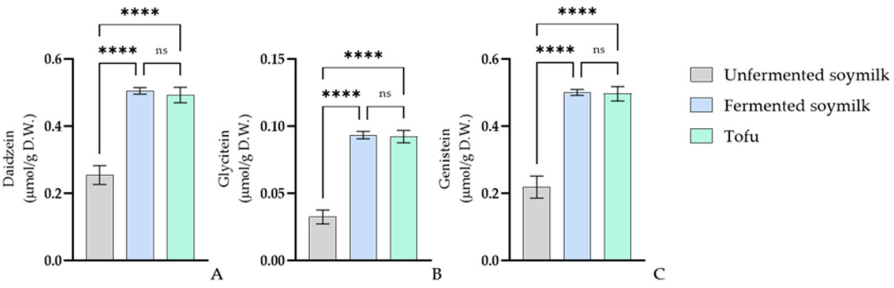

Figure 3. Aglycone isoflavones ((**A**): daidzein; (**B**): glycitein; (**C**): genistein) in unfermented soymilk, fermented soymilk (24 h) and tofu (21 days) using *Lp. plantarum* LP95 as starter. Error bars indicate the standard deviation of the mean (n = 3). ns: not significant, ****: $p < 0.0001$.

3.5. Free Amino Acid Profiles

Twenty-two free L-amino acids were detected and quantified in the unfermented, fermented soymilk (after 24 h) and tofu (after 21 days). Results are reported in Table 2. In the unfermented soymilk, the most abundant amino acids, were γ-aminobutyric acid (GABA; 8.21 ± 1.53), arginine (6.01 ± 1.23), proline (5.60 ± 0.49) and threonine (4.67 ± 0.90). After fermentation of the soymilk, no significant variation was observed in the amounts of arginine, gamma aminobutyric acid (GABA), histidine, lysine, mono-ethanolamine, ornithine and threonine. After the maturation phase of Tofu (21 days) the content of the amino acids did not undergo significant changes compared to fermented soymilk, except for a slight significant decrease in glutamine and proline.

Table 2. L-Amino acids content in unfermented soymilk, fermented soymilk (24 h) and tofu (21 days) using *Lp. plantarum* LP95 as starter. All values are expressed as the mean ± standard deviation (n = 3).

L-Amino Acids (μmol/g D.W.)	Unfermented Soymilk	Fermented Soymilk	Tofu
Alanine	3.03 ± 0.52 [a]	0.50 ± 0.06 [b]	0.64 ± 0.09 [b]
Arginine	6.01 ± 1.23 [a]	7.11 ± 1.11 [a]	4.93± 1.05 [a]
Asparagine	10.8 ± 1.62 [a]	2.91 ± 0.42 [b]	2.18 ± 0.39 [b]
Aspartic acid	1.20 ± 0.20 [a]	0.07 ± 0.01 [b]	0.08 ± 0.01 [b]
γ-aminobutyric acid	8.21 ± 1.53 [a]	8.87 ± 1.65 [a]	6.75 ± 1.16 [a]
Glutamine	0.15 ± 0.01 [a]	0.12 ± 0.03 [a]	0.08 ± 0.04 [b]
Glutamic acid	2.73 ± 0.43 [a]	0.00 ± 0.01 [b]	0.00 ± 0.01 [b]
Glycine	0.80 ± 0.11 [a]	0.28 ± 0.11 [b]	0.22 ± 0.09 [b]
Histidine	0.95 ± 0.15 [a]	1.02 ± 0.23 [a]	0.78 ± 0.20 [a]
lsoleucine	0.85 ± 0.13 [a]	0.09 ± 0.01 [b]	0.07 ± 0.01 [b]
Leucine	1.33 ± 0.21 [a]	0.15 ± 0.02 [b]	0.13 ± 0.02 [b]
Lysine	0.76 ± 0.14 [a]	1.13 ± 0.41 [a]	0.98 ± 0.29 [a]
Monoethanolamine	0.84 ± 0.15 [a]	0.96 ± 0.16 [a]	0.79 ± 0.12 [a]
Methionine	0.32 ± 0.04 [a]	0.10 ± 0.02 [b]	0.07 ± 0.01 [b]
Ornithine	0.45 ± 0.05 [a]	0.59 ± 0.04 [a]	0.48 ± 0.13 [a]
Phenylalanine	1.17 ± 0.20 [a]	0.02 ± 0.01 [b]	0.02 ± 0.01 [b]
Proline	5.60 ± 0.49 [a]	5.70 ± 0.85 [a]	3.85 ± 1.00 [b]
Serine	1.05 ± 0.09 [a]	0.17 ± 0.03 [b]	0.15 ± 0.03 [b]
Threonine	4.67 ± 0.90 [a]	5.74 ± 1.10 [a]	4.46 ± 0.71 [a]
Tryptophan	1.65 ± 0.29 [a]	0.15 ± 0.04 [b]	0.13 ± 0.04 [b]
Tyrosine	0.93 ± 0.13 [a]	0.02 ± 0.01 [b]	0.01 ± 0.00 [b]
Valine	1.36 ± 0.20 [a]	0.08 ± 0.01 [b]	0.06 ± 0.01 [b]

Different lowercase letters (a,b) in each row indicate significant differences ($p < 0.05$).

3.6. Antioxidant Activity

The antioxidant activity in the unfermented soymilk, fermented soymilk (after 24 h) and tofu samples (after 21 days), was assayed by applying the ABTS and DPPH methods. The two methods applied showed differences in the sensitivity of identification of the antioxidant compounds: ABTS scavenging activity progressively decreased, while the DPPH scavenging activity increased during the fermentation phase (24 h), as shown in Table 3. In detail, the ABTS assay showed a significant decrease of TEAC (mg Trolox Eq/g D.W.) during production (from about 9% in fermented soymilk to about 30% in tofu). The DPPH from an initial value of about 0.13 mg Trolox Eq/g D.W. showed a significant increase after fermentation (about 40%) and then remained unchanged in tofu, after 21 days.

Table 3. Antioxidant activity and total phenolic content (TPC) in unfermented soymilk, fermented soymilk (24 h) and tofu (21 days) using *Lp. plantarum* LP95 as starter. All values are expressed as the mean ± standard deviation (n = 3).

	Unfermented Soymilk	Fermented Soymilk	Tofu
* ABTS	0.69 ± 0.02 [a]	0.63 ± 0.02 [b]	0.48 ± 0.01 [c]
* DPPH	0.13 ± 0.02 [b]	0.18 ± 0.01 [a]	0.17 ± 0.01 [a]
** TPC	8.27 ± 0.15 [c]	9.95 ± 0.19 [a]	9.63 ± 0.26 [b]

* (mg Trolox Eq./g D.W.); ** (mg Gallic acid Eq./g D.W.). Different lowercase letters (a–c) in each row indicate significant differences ($p < 0.05$).

3.7. Total Phenolic Content

The total phenolic content (TPC) values, expressed as mg Gallic acid Eq./g D.W. are reported in Table 3. After soymilk fermentation, the TPC value increased significantly to about 20% and then remained almost unchanged in tofu.

4. Discussion

As reported by several studies, *Lp. plantarum* is one of the most widely used LAB species as a starter for the fermentation of plant-based products [31–33], including soybean products [34,35]. Indeed, *Lp. plantarum* LP95 showed a marked adaptability to the soymilk substrate and good fermentation kinetics towards sugars naturally present in soymilk. Importantly, the ability to catabolize the different sugars in soymilk is strictly dependent on the LAB strain used as a starter [36]. As above reported, the used *Lp. plantarum* LP95 is able to ferment sucrose, the main disaccharide found in soymilk. This LAB strain shows a rapid ability to completely ferment glucose and fructose after 24 h. This means that tofu made from fermented soymilk with *Lp. plantarum* LP95 will be low in monosaccharides and disaccharides, making it a functional product with a low sugar content that can be used in low-calorie diets. In addition, it should be emphasized that the fermentation of soymilk by *Lp. plantarum* LP95 results in a complete metabolization of galactose (C-4 glucose epimer) which, if introduced into the diet, after digestion, can be directly absorbed and enter the bloodstream to reach the target tissues where it contributes to energy production, glycosylation and other important metabolic functions [37]. However, it has been reported that an excess of D-galactose can have a deleterious effects in murine models [38], causing an increase in the metabolic rates in the body and a high accumulation of free radicals, thus leading to damage in DNA, proteins, cell membrane lipids, nerves, mitochondria, and to an increase in cognitive decline [39]. Interestingly, *Lp. plantarum* LP95 was unable to metabolize stachyose and raffinose, probably due to lack of α-galactosidase. The presence of stachyose and raffinose in Tofu can take on a health aspect in humans. In fact, these galacto-oligosaccharides (GOS), as prebiotics, improve the balance of the gastrointestinal microbiota, promoting the growth of beneficial bacteria and inhibiting the growth of pathogens [40–43].

Our results showed a good survival capacity of *Lp. plantarum* after 21 days of storage at 4 °C of tofu. In this regard, it is important to emphasize that the consumption of fermented foods rich in probiotic bacteria positively modifies the gut microbiota by increasing the number of beneficial bacteria and lowering the number of potential pathogens [44].

Previous works reported that the fermentation of soymilk with LAB promotes the formation of bioactive compounds, like peptides, total isoflavone aglycone, γ-aminobutyric acid, and phenols [36]. Moreover, a positive relationship between the LAB growth rate and the isoflavone conversion has been shown, since growth rate is related to β-glucosidase activity, which converts isoflavone glycosides into aglycones [45]. As is reported in this study, β-glucosidase activity of *Lp. plantarum* LP95 increased the content of isoflavone aglycones in fermented soymilk. In particular, the levels of daidzein, genistein and glycitein detected in the fermented soymilk and tofu increased by 50, 65 and 56%, respectively, and remained unchanged until 21th day of storage at 4 °C. The detection of isoflavones after 21 days strengthens the suitability of the *Lp. plantarum* LP95 strain to be used as a starter, as it does not further catabolize isoflavone aglycones. Dietary isoflavones have been well study for their health benefits; several reports, in fact, have demonstrated their bioactivities, such as anti-cancer, anti-obesity, anti-inflammation, anti-diabetes activities, gut microbiota regulation and osteoporosis prevention [46].

Overall, the content of the free L-amino acids, including GABA, in tofu produced by *Lp. plantarum* LP95, did not change significantly compared to unfermented and fermented soymilk. GABA is a non-protein amino acid, widely distributed in an variety of organisms, such as animals, plants, algae, bacteria and fungi [18], with established physiological functions, including neurotransmission, development of hypotension, and diuretic and tranquilizing actions. These evidences result in a high nutritional value of the obtained tofu, comparable to that of other soy products [36,47].

As described above, *Lp. plantarum* LP95, after the fermentation phase, significantly increased the total phenolic content, which remained unchanged up to 21 days of storage of the tofu. This is another significant aspect from a nutritional and functional point of view because phenolic compounds, mainly derived from plants, have been reported to exhibit

antioxidative, immunomodulatory, antimicrobial, antiviral, antihypertensive activity and anticancer properties [48]. Nevertheless, the majority of common phenolic chemicals are usually found in insoluble forms that are difficult to use when bonded with carbohydrates and proteins [49,50]. Due to the split of these insoluble complex by microbial enzymes, such as cellulase, hemicellulase, amylase, protease, and pectinase, fermentation may promote the release of insoluble compounds, thereby increasing the functional qualities of the food [49]. It was shown that the proteolytic activity of a number of LAB increased the amount of phenolic and isoflavones, as well as the antioxidant capacity in fermented soymilk [51].

As far as the antioxidant activity is concerned, our results show a different trend, depending on the used method. Since the ABTS and the DPPH methods have a different sensitivity towards different antioxidant compounds, this may explain the different observed TEAC values. This different sensitivity has been previously described and observed in studies conducted on other plant products [52,53].

5. Conclusions

The reported study elucidates the fermentation kinetics of *Lp. plantarum* LP95 in the soymilk substrate, demonstrating that it is able to rapidly ferment monosaccharides and disaccharides naturally present in soymilk after 24 h, consequently lowering the glycemic content and leaving the content of GOS, considered important prebiotics, unchanged. Furthermore, the use of *Lp. plantarum* LP95 proved useful in the production of fermented tofu, as it did not alter (degrade or bio-convert) the content of the isoflavone aglycones within 21 days and did not substantially change the content of free L-amino acids, including GABA.

In addition, *Lp. plantarum* LP95 showed a good viability after 21 days of tofu storage at 4 °C. The results obtained highlight the suitability of this LAB strain to be used as a microbial culture able to play a pro-technological role in the production of fermented tofu with intrinsic nutritional and functional properties. In the future, it would also be interesting to evaluate the organoleptic characteristic of soybean products fermented by this bacterium.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12061093/s1, Table S1: Changes in pH of soymilk during the fermentation by *Lp. plantarum* LP95; Table S2: Variations in sugars content of soymilk during the fermentation by *Lp. plantarum* LP95; Table S3: Aglycone isoflavones (daidzein, glycitein, and genistein) in unfermented soymilk, fermented soymilk (24 h) and tofu (21 days) using *Lp. plantarum* LP95 as starter.

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