

Article

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

Francesco Letizia ¹, Giovanna Marta Fusco ², Alessandra Fratianni ^{1,*}, Ilenia Gaeta ¹, Petronia Carillo ², Maria Cristina Messia ¹ and Massimo Iorizzo ¹

¹ Department of Agriculture, Environmental and Food Sciences, University of Molise, Via De Sanctis, 86100 Campobasso, Italy; f.letizia@studenti.unimol.it (F.L.); i.gaeta@studenti.unimol.it (I.G.); messia@unimol.it (M.C.M.); iorizzo@unimol.it (M.I.)

² Department of Environmental Biological and Pharmaceutical Sciences and Technologies, University of Campania "Luigi Vanvitelli", Via Vivaldi 43, 81100 Caserta, Italy; giovannamarta.fusco@unicampania.it (G.M.F.); petronia.carillo@unicampania.it (P.C.)

* Correspondence: fratianni@unimol.it

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.

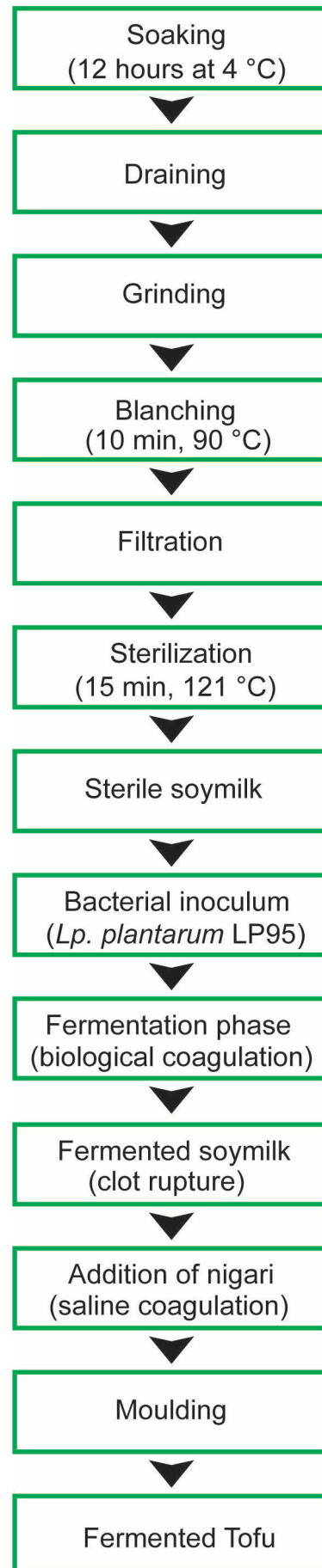


Figure 1. Flow chart of fermented tofu production.

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 Am-atayakul 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).

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

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 \times 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 \times 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 DDU-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^{•+}), by decolorization of the 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS^{•+}), 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^{•+}) were produced by reacting the ABTS methanol solution with 2.45 mM potassium persulfate. The ABTS^{•+} 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[•]) 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[•] 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 \pm standard deviation (\pm 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 \pm standard deviation ($n = 3$).

	Days of Soymilk Fermentation			Days of Tofu Storage	
	0	1	7	14	21
Viable cell count	8.07 \pm 0.06 ^b	9.52 \pm 0.11 ^a	7.76 \pm 0.29 ^b	6.00 \pm 0.50 ^c	5.83 \pm 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 \pm 0.10%, 3.73 \pm 0.49% and 4.1 \pm 0.93% respectively, while the a_w of the same samples was 0.26 \pm 0.05, 0.21 \pm 0.02 and 0.16 \pm 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.

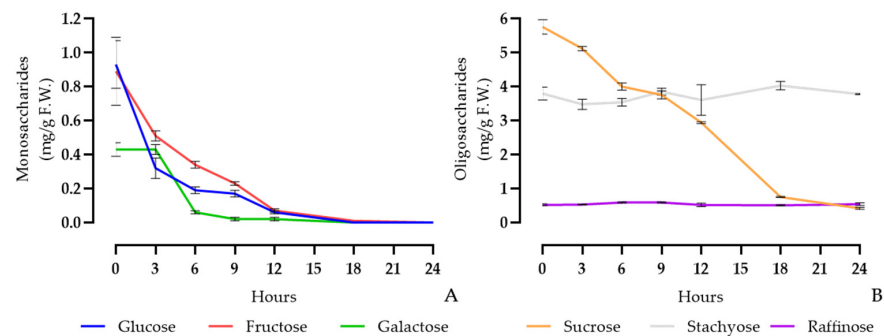


Figure 2. Quantitative changes in sugars during fermentation of soymilk using *Lp. plantarum* LP95 as starter: glucose, fructose, galactose (A); sucrose, stachyose and raffinose (B). Error bars indicate the standard deviation of the mean (n = 3).

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 $\mu\text{mol/g}$ on dry weight; D.W., respectively), while their aglycosylated forms were daidzein, glycitein, and genistein (0.25 ± 0.03 , 0.03 ± 0.01 , 0.21 ± 0.03 $\mu\text{mol/g}$ 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 $\mu\text{mol/g}$ D.W., respectively.

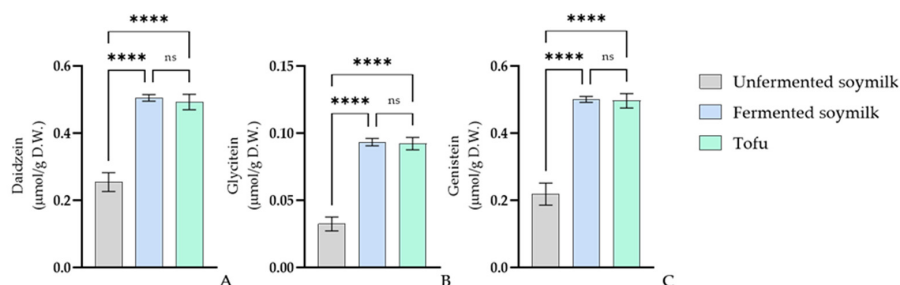


Figure 3. Aglycone isoflavones ((A): daidzein; (B): glycitein; (C): genistein) in unfermented soymilk, fermented soymilk (24 h) and tofu (21 days) using *Lp. plantarum* LP95 as starter. Error bars indicate the standard deviation of the mean (n = 3). ns: not significant, ****: $p < 0.0001$.

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 \pm standard deviation (n = 3).

L-Amino Acids ($\mu\text{mol/g D.W.}$)	Unfermented Soymilk	Fermented Soymilk	Tofu
Alanine	3.03 \pm 0.52 ^a	0.50 \pm 0.06 ^b	0.64 \pm 0.09 ^b
Arginine	6.01 \pm 1.23 ^a	7.11 \pm 1.11 ^a	4.93 \pm 1.05 ^a
Asparagine	10.8 \pm 1.62 ^a	2.91 \pm 0.42 ^b	2.18 \pm 0.39 ^b
Aspartic acid	1.20 \pm 0.20 ^a	0.07 \pm 0.01 ^b	0.08 \pm 0.01 ^b
γ -aminobutyric acid	8.21 \pm 1.53 ^a	8.87 \pm 1.65 ^a	6.75 \pm 1.16 ^a
Glutamine	0.15 \pm 0.01 ^a	0.12 \pm 0.03 ^a	0.08 \pm 0.04 ^b
Glutamic acid	2.73 \pm 0.43 ^a	0.00 \pm 0.01 ^b	0.00 \pm 0.01 ^b
Glycine	0.80 \pm 0.11 ^a	0.28 \pm 0.11 ^b	0.22 \pm 0.09 ^b
Histidine	0.95 \pm 0.15 ^a	1.02 \pm 0.23 ^a	0.78 \pm 0.20 ^a
Isoleucine	0.85 \pm 0.13 ^a	0.09 \pm 0.01 ^b	0.07 \pm 0.01 ^b
Leucine	1.33 \pm 0.21 ^a	0.15 \pm 0.02 ^b	0.13 \pm 0.02 ^b
Lysine	0.76 \pm 0.14 ^a	1.13 \pm 0.41 ^a	0.98 \pm 0.29 ^a
Monoethanolamine	0.84 \pm 0.15 ^a	0.96 \pm 0.16 ^a	0.79 \pm 0.12 ^a
Methionine	0.32 \pm 0.04 ^a	0.10 \pm 0.02 ^b	0.07 \pm 0.01 ^b
Ornithine	0.45 \pm 0.05 ^a	0.59 \pm 0.04 ^a	0.48 \pm 0.13 ^a
Phenylalanine	1.17 \pm 0.20 ^a	0.02 \pm 0.01 ^b	0.02 \pm 0.01 ^b
Proline	5.60 \pm 0.49 ^a	5.70 \pm 0.85 ^a	3.85 \pm 1.00 ^b
Serine	1.05 \pm 0.09 ^a	0.17 \pm 0.03 ^b	0.15 \pm 0.03 ^b
Threonine	4.67 \pm 0.90 ^a	5.74 \pm 1.10 ^a	4.46 \pm 0.71 ^a
Tryptophan	1.65 \pm 0.29 ^a	0.15 \pm 0.04 ^b	0.13 \pm 0.04 ^b
Tyrosine	0.93 \pm 0.13 ^a	0.02 \pm 0.01 ^b	0.01 \pm 0.00 ^b
Valine	1.36 \pm 0.20 ^a	0.08 \pm 0.01 ^b	0.06 \pm 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 \pm standard deviation (n = 3).

	Unfermented Soymilk	Fermented Soymilk	Tofu
* ABTS	0.69 \pm 0.02 ^a	0.63 \pm 0.02 ^b	0.48 \pm 0.01 ^c
* DPPH	0.13 \pm 0.02 ^b	0.18 \pm 0.01 ^a	0.17 \pm 0.01 ^a
** TPC	8.27 \pm 0.15 ^c	9.95 \pm 0.19 ^a	9.63 \pm 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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