Heliyon 9 (2023) e14991

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Fermented soy beverages as vehicle of probiotic lactobacilli strains and source of bioactive isoflavones: A potential double functional effect

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ARTICLE INFO

Keywords:
Daidzein
Genistein
Bifidobacterium
Lacticaseibacillus
Probiotics

ABSTRACT

Soy beverages can be a source of bioactive isoflavones, with potential human health benefits. In this work, the suitability of three *Lacticaseibacillus* and three *Bifidobacterium* probiotic strains as functional starters for soy beverage fermentation were evaluated, alongside with the effect of refrigerated storage on the viability of the strains and the isoflavone composition of the fermented beverages. The three bifidobacteria strains suffered a decrease in their viability during refrigeration and only *Bifidobacterium breve* INIA P734 produced high concentrations of bioactive isoflavones. Meanwhile, *L. rhamnosus* GG and *L. rhamnosus* INIA P344 produced high levels of aglycones and, with *L. paracasei* INIA P272, maintained their viability during the refrigeration period, constituting promising starters to obtain functional soy beverages that could gather the benefits of the bioactive isoflavone aglycones and the probiotic strains. Moreover, the three lactobacilli caused an increase in the antioxidant capacity of the fermented beverages, which was maintained over the refrigerated storage.

1. Introduction

The nutritional composition of soy beverage makes it a rich source of proteins of vegetal origin with low fat content, free from lactose and cholesterol and rich in bioactive compounds; hence, this beverage is considered a healthy food [1]. Soy is a source of isoflavones, compounds classified as phytoestrogens linked to beneficial effects in health [2]. Isoflavones appear in soybeans predominantly in the form of β-glycosides and their acetyl- and malonyl-conjugates, while soy beverage contains mainly β-glycosides daidzin and genistin due to thermal processing [3]. However, the β-glycosides are poorly absorbed after ingestion unless they are hydrolyzed into aglycones (daidzein and genistein) [4,5]. This reaction is carried out by intestinal or bacterial enzymes, however, the final amount of aglycones and subsequent metabolites reached in the organisms is subjected to high interindividual variability [6–8].

Fermentation with aglycone-producing bacterial strains as starters is a way to increase the potential health-promoting effects of soy products [9,10]. In this regard, the beneficial effects of fermented soy beverages have been linked with a positive impact in reducing osteoporosis, hypercholesterolemia, obesity, carcinogenesis and tumour growth [11]. Lactic acid bacteria (LAB) and bifidobacteria can be used to produce fermented soy beverages [11,12]. Besides to an improvement in the sensory characteristics of soy beverage,

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https://doi.org/10.1016/j.heliyon.2023.e14991
Received 16 September 2022; Received in revised form 14 February 2023; Accepted 23 March 2023
Available online 3 April 2023

fermentation can increase the levels of aglycones present in the product, so, choosing a functional starter strain should entail the assessing of its ability to transform the isoflavones into their aglycone forms [9]. Additionally, fermented soy beverages can be a suited matrix for probiotic microorganisms [13,14]. However, none of these works have studied the production of bioactive isoflavones by probiotic strains. So, the enrichment in bioactive isoflavones in fermented foods through probiotic strains is an unexplored field.

LAB and *Bifidobacterium* strains tested in the following study were selected based on their probiotic potential [15–18]. *Lacticaseibacillus paracasei* INIA P272 and *Lacticaseibacillus rhamnosus* INIA P344 presented good tolerance to gastrointestinal conditions, high adherence to intestinal epithelial cells, a broad spectrum of antimicrobial activity and the ability to coaggregate with various pathogens, and proved their safety by the absence of virulence, pathogenicity and antibiotic resistance genes [17,18]. *Bifidobacterium breve* INIA P734 and *Bifidobacterium longum* INIA P132 also demonstrated a great survival to gastrointestinal conditions, were able to form biofilms and did not present pathogenicity and antibiotic resistance genes [15,16,19], and *B. longum* INIA P132 was also able to produce exopolisacharides with immune-modulatory effect [15]. Moreover, some of these lactobacilli and bifidobacteria strains have previously shown good survival in a dairy fermented food, such as cheese [20,21]. Therefore, these bacteria could be good candidates for the development of fermented soy beverages enriched in bioactive isoflavones and serving as probiotics, thanks to their probiotic and technological characteristics.

Thus, we analyzed the suitability of the aforementioned strains from our collection, as well as of the commercial probiotic strains *L. rhamnosus* GG [22] and *B. longum* BB536 [23] as fermentation starters and bioactive isoflavones producers in soy beverages. Additionally, the survival of these strains, and the antioxidant capacity and the phenolic content of the beverages were studied after the soy beverage fermentation and during 28 days refrigerated storage. After the fermentation process, fermented soy beverages are usually conserved under refrigeration during their self-live, what can affect the viability of the starter strain and their isoflavone composition [24]. This last aspect has been scarcely studied in scientific literature [25] and therefore, it has been addressed as an important issue in the present work.

2. Material and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study, their sources and characteristics are listed in Table 1. *Bifidobacterium* strains were cultivated in MRS broth (BD; Becton, Dickinson &Co., Le Pont de Claix, France) supplemented with 0.5 g/L L-cysteine (Sigma-Aldrich, St Louis, MO), at 37 °C under anaerobic conditions (10% H_2, 10% CO_2 y 80% N_2. Whitley DG250 Anaerobic workstation, Don Whitley Scientific Ltd., Shipley, UK). Lactobacilli were routinely cultivated in MRS broth at 37 °C under anaerobic conditions.

2.2. Fermentation of soy beverage and refrigeration

A commercial UHT soy beverage (Vital, DIA, Madrid, Spain) was purchased from a local supermarket. The composition of the beverage, as state in the label is: water, soy beans (13%), fructose, tricalcium phosphate, flavoring, salt, gellan gum and vitamins (A and D); and the nutritional values over 100 mL are: energy 43 kcal, fats 1.7 g (of which saturates 0.3 g), carbohydrates 3.7 g (of which sugars 3.0 g), proteins 3.1 g, salt 0.1 g, vitamin A 120 μg, vitamin D 0.75 μg, calcium 120 mg.

First, stock inoculums of all the strains used as starters for the soy beverage fermentation were prepared as described by Ruiz de la Bastida et al. [12] and conserved at −80 °C until use. The viability of the frozen inoculums was determined previously to stablish the amount of inoculum to be used for each strain. An inoculum of approximatively 0.1% was used to obtain an initial concentration of 6.5–7.0 log cfu/ml at the beginning of the soy beverage fermentation. The inoculated soy beverages were incubated for 24 h at 37 °C under anaerobic conditions. A non-inoculated control was included in each one of the two independent experiments realized.

After incubation, fermented and control soy beverages were aliquoted in 11 mL volume in sterile tubes and store at 5 ± 1 °C during 28 days. Bacterial counts and pH determinations were performed before and after the fermentation, and during the refrigerated storage at intervals of 7 days. pH was measured in duplicate by means of a Crison pH meter (model GPL 22, Crison Instruments, Barcelona, Spain). For microbial counts, decimal dilutions were prepared in sterile 0.1% peptone water and plate out on duplicate plates. *Bifidobacterium* counts were performed on Reinforced Clostridial Medium (BD; Becton, Dickinson & Co.) agar plates incubated at 37 °C for

Table 1
Bacterial strains tested in this work.

Strain	Source	Characteristics	References
Bifidobacterium breve INIA P734	Breast-feed infant faeces	Probiotic traits, potential adjunct culture in cheese	Langa et al., 2020; Peirotén et al., 2018; Rodrigo-Torres et al., 2021
Bifidobacterium longum INIA P132	Breast-feed infant faeces	Probiotic traits, exopolisacharides with immune-modulatory effect	Llamas-Arribas et al., 2019; Rodríguez et al., 2012
Bifidobacterium longum BB536	Commercial strain	Probiotic strain	Wong et al., 2019
Lacticaseibacillus paracasei INIA P272	Breast-feed infant faeces	Probiotic traits,	Rodrigo-Torres et al., 2022; Rodríguez et al., 2012
Lacticaseibacillus rhamnosus INIA P344	Breast-feed infant faeces	Probiotic traits,	Rodrigo-Torres et al., 2022; Rodríguez et al., 2012
Lacticaseibacillus rhamnosus GG	Commercial strain	Probiotic strain	Capurso 2019

48 h under anaerobic conditions. In the case of lactobacilli, MRS agar plates were used under the same conditions. Samples for isoflavones quantification were taken periodically and frozen at −20 °C until analysis.

2.3. Extraction of isoflavones from fermented soy beverages

Samples obtained from the fermented and control soy beverages were submitted to HPLC analysis to assess the evolution of the isoflavones concentration after fermentation and during refrigerated storage. Isoflavones were extracted in duplicate from the beverages following the official AOAC method [26]. Briefly, the reaction mixture contained 1 mL of the fermented or control sample and 500 μL of acetonitrile. The mixture was shaken vigorously for 60 min and centrifuged for 10 min at 13,500 rpm. The supernatant was filtered through a 0.22 μm PVDF membrane filter (Teknokroma, Sant Cugat del Vallés, Spain) and stored at −20 °C until HPLC analysis.

2.4. Quantification of isoflavones in fermented soy beverages

The extracted samples were analyzed twice by HPLC-PAD according to Gaya et al. [27]. In brief, analysis was carried out on a HPLC-PAD Beckman System Gold (Beckman Coulter Inc., Fullerton, CA, USA), with a reverse phase Nova-Pak C18 column (300 × 3.9 mm, 4 μm) (Waters, Barcelona, Spain) and a dynamic gradient of acetic acid/acetonitrile. Detection was performed in a diode array detector module 168 by scanning from 210 to 400 nm with an acquisition speed of 1 s. Quantification was carried out by means of external standard calibration curves. Daidzin, daidzein, equol, genistin and genistein were purchased from LC Laboratories (Woburn, MA). Dihydrodaidzein and dihydrogenistein were purchased from Toronto Research Chemicals (Toronto, Canada). Stock solutions of isoflavones were prepared in DMSO (Sigma-Aldrich) and the concentrations tested were prepared in triplicate in soy beverage and subjected to the same extraction process than the samples to obtain the calibration curve.

2.5. Determination of antioxidant activity and phenolic compounds

Antioxidant activity was determined by the ferric reducing antioxidant power (FRAP) method according to del Olmo et al. [28]. In brief, supernatants (30 μL) were incubated at 37 °C for 60 min with Milli-Q water (100 μL) and FRAP reagent (900 μL) containing 2,4, 6-tris(2-pyridyl)-s-triazine (TPTZ, Sigma-Aldrich) and $FeCl_3$ $6H_2O$. The absorbance was read at 595 nm to monitor Fe^{3+} reduction and the formation of a coloured TPTZ-Fe^{2+} complex. A standard curve was obtained using Trolox (Sigma-Aldrich) and the results were expressed as trolox equivalents (mg/ml). Assays were performed in triplicate.

Phenolic compounds were determined by the Folin–Ciocalteau colorimetric method adapted from Ferraces-Casais et al. [29]. The fermented broths (100 μL) were mixed with a 6% Na_2CO_3 aqueous solution (1 ml) and the Folin–Ciocalteau reagent (100 μL). The mixture was incubated for 1 h at room temperature, and the absorbance was read at 740 nm. Quantification was carried out using a gallic acid (Sigma-Aldrich) standard curve and the results expressed as mg/ml gallic acid equivalents.

2.6. Statistical analysis

Statistical analysis of the data was performed using of SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, USA) by ANOVA using a general linear model (GLM). Comparison of means ($P < 0.01$) was carried out by Tukey test.

3. Results

3.1. Bacterial counts and pH after fermentation and during refrigeration

All the strains tested as starters for the soy beverage fermentation showed good growth after 24 h incubation, increasing their levels between 1.7 and 2.8 log units and reaching counts between 8.6 and 9.7 log cfu/mL (Table 2). *L. paracasei* INIA P272, *L. rhamnosus* INIA P344 and *L. rhamnosus* GG reached the highest counts after 24 h incubation, followed by *B. breve* INIA P734.

The three lactobacilli tested maintained their levels over 9 log units during the storage for 28 days at 5 °C, while the bifidobacteria strains maintained high counts until the seventh day of refrigeration followed by a continuous drop in their counts during the

Table 2
Counts (log cfu/mL) of selected strains in fermented soy beverage, before and after fermentation, and during refrigerated storage.

	Fermentation		Refrigerated storage			
Strain	Initial	24 h	7 days	14 days	21 days	28 days
B. breve INIA P734	7.14 ± 0.03^{e}	9.14 ± 0.10^{hi}	8.54 ± 0.19^{gh}	6.14 ± 0.71^{c}	4.54 ± 0.33^{b}	2.14 ± 0.38^{a}
B. longum INIA P132	6.38 ± 0.03^{cd}	8.59 ± 0.14^{gh}	7.86 ± 0.21^{f}	6.14 ± 0.41^{c}	4.37 ± 0.45^{b}	2.56 ± 0.36^{a}
B. longum BB536	6.45 ± 0.05^{cd}	8.83 ± 0.04^{gh}	8.79 ± 0.03^{gh}	8.36 ± 0.10^{fg}	6.87 ± 0.17^{de}	4.78 ± 0.07^{b}
L. paracasei INIA P272	6.81 ± 0.03^{de}	9.61 ± 0.04^{i}	9.68 ± 0.03^{i}	9.56 ± 0.06^{i}	9.64 ± 0.02^{i}	9.63 ± 0.07^{i}
L. rhamnosus INIA P344	6.86 ± 0.01^{de}	9.67 ± 0.10^{i}	9.64 ± 0.08^{i}	9.67 ± 0.02^{i}	9.71 ± 0.02^{i}	9.67 ± 0.03^{i}
L. rhamnosus GG	6.85 ± 0.15^{de}	9.49 ± 0.11^{i}	9.59 ± 0.02^{i}	9.58 ± 0.03^{i}	9.53 ± 0.08^{i}	9.55 ± 0.07^{i}

[a–i]Different superscript letter indicate statistically significant differences ($P < 0.01$, Tukey test). Values are mean ± SD.

refrigerated storage. *B. longum* BB536 was able to maintain its viable counts over 8 log units until day 14 and reach similar levels to the initial inoculum at day 21; at the end of the refrigerated storage, its counts decreased until 4.78 log units, showing significantly ($P < 0.01$) higher levels than those of the other two bifidobacteria. However, the other two bifidobacteria showed a decrease from the first week, but maintained good counts until the seventh day of refrigeration, being *B. breve* INIA P734 the one that registered higher levels of viable on day 7 (Table 2). Viability loss of the bifidobacteria strains tested was between 4 and 7 log units at the end of the storage period.

The higher growth of *L. paracasei* INIA P272, *L. rhamnosus* INIA P344 and *L. rhamnosus* GG was accompanied with a higher drop in pH, reaching pH values of 3.8 in these fermented soy beverages after 24 h fermentation (Table 3). The three bifidobacteria recorded significantly ($P < 0.01$) lower acidification values of the soy beverages after fermentation compared to the lactobacilli strains, reaching pH levels higher than 4.7. *B. longum* INIA P132 and *B. breve* INIA P734 strains obtained lower pH levels after 24 h of fermentation compared to *B. longum* BB536, with a pH of 5.1 (Table 3). The pH was generally stable along the refrigerated storage of the fermented soy beverages; however, several strains, including the bifidobacteria *B. longum* BB536, showed a slight tendency towards decreasing the pH over time, although the differences were between 0.30 units.

3.2. Isoflavone metabolism

The three lactobacilli strains and *B. breve* INIA P734 transformed the isoflavone glycosides into aglycones during the fermentation of the soy beverage (Table 4). The increment of daidzein levels in the fermented soy beverages for these strains ranged between 2.2 and 8.0 fold compared to the daidzein present in control, being *L. rhamnosus* INIA P344 the strain showing the highest levels of daidzein after 24 h fermentation. As for genistein, increment folds went from 3.6 to 15.1 compared to the control soy beverage, being *L. rhamnosus* INIA P344 and *L. rhamnosus* GG the strains with the highest production. The two *L. rhamnosus* strains also consumed most of the glycosides, reducing their levels an 82.9% and a 72.1%, respectively, as compared to the total daidzin plus genistin present in the control beverage. While *L. paracasei* INIA P272 showed a lower metabolism of glycosylated isoflavones during the fermentation of the beverages compared to the other two lactobacilli strains, *B. breve* INIA P734 stood out as the third strain with the highest concentrations of aglycones after 24 h of fermentation. However, both *B. longum* INIA P132 and *B. longum* BB536 strains were not able to metabolize these glycosides present in the soy beverage during fermentation or throughout the refrigeration period, showing significantly ($P < 0.01$) similar levels as the control soy beverage. The levels of isoflavones did not change during the 28 days storage in cold in these beverages, except for the one with *B. longum* INIA P132 that showed a significant ($P < 0.01$) decrease of in the daidzin levels; genistin was the compound with the highest presence, followed by daidzin, and with minimal concentrations of daidzein and genistein.

Over the refrigerated storage, the levels of aglycones present in the beverages fermented with the four aglycone-producing strains were maintained or even increased (Table 4). At the end of the storage period, the three lactobacilli strains and *B. breve* INIA P734 showed significant ($P < 0.01$) rise in daidzein concentrations. The highest levels of this aglycone were obtained in the beverage fermented with *L. rhamnosus* INIA P344, followed by the one with *B. breve* INIA P734, which almost duplicated the content of daidzein during the storage. Significant ($P < 0.01$) increments in genistein during refrigerated storage were observed in the beverages fermented with *B. breve* INIA P734 and *L. paracasei* INIA P272, while for the two strains showing the highest genistein, *L. rhamnosus* INIA P344 and *L. rhamnosus* GG, that increase was not significantly. The incremental trend of aglycones during the refrigerated storage was corroborated by analysing intermediate samples taken at 14 days of storage, which showed intermediated levels of aglycones to those observed in days 1 and 28 of storage (Fig. 1). In terms of glycosides metabolization, at the end of the refrigerated storage, *B. breve* INIA P734 and the two *L. rhamnosus* strains had consumed between 76.1 and 85.6% of the sum of genistin and daidzin as compared with the control soy beverage. *B. breve* INIA P734 was the strain with the highest capacity to gradually metabolize glycosides during the refrigeration period, reflected in the levels of aglycones recorded at the days 14 and 28 (Fig. 1). This bifidobacteria obtained the highest rise in aglycone levels on day 28 of storage, despite its decrease in viable counts from day 7 of refrigeration (Table 2), and consequently there was a significant decrease in glycosides (Table 3). As a result, the increment in aglycones at the end of the storage for fermented soy beverages with *B. breve* INIA P734 and both *L. rhamnosus* ranged between 11.4 and 13.0 folds compared with the aglycones in the control ones. Subsequent metabolites of aglycones, i.e., equol, dihydrodaidzein and dihydrogenistein, were not detected in any of the fermented soy beverages.

Table 3
pH of fermented soy beverages after fermentation and during refrigerated storage.

	Fermentation		Refrigerated storage			
Strain	Initial	24 h	7 days	14 days	21 days	28 days
Control soy beverage	7.12 ± 0.03^{i}	7.05 ± 0.08^{hi}	6.85 ± 0.04^{gh}	6.81 ± 0.06^{gh}	6.82 ± 0.04^{gh}	6.80 ± 0.02^{gh}
B. breve INIA P734	–	4.80 ± 0.13^{de}	4.77 ± 0.01^{de}	4.76 ± 0.03^{de}	4.81 ± 0.05^{de}	4.80 ± 0.08^{de}
B. longum INIA P132	–	4.78 ± 0.13^{de}	4.78 ± 0.02^{de}	4.79 ± 0.02^{de}	4.87 ± 0.08^{ef}	4.82 ± 0.07^{de}
B. longum BB536	–	5.11 ± 0.15^{f}	4.87 ± 0.03^{ef}	4.80 ± 0.07^{de}	4.78 ± 0.05^{de}	4.77 ± 0.07^{de}
L. paracasei INIA P272	–	3.82 ± 0.11^{b}	3.75 ± 0.05^{b}	3.69 ± 0.04^{ab}	3.64 ± 0.10^{ab}	3.46 ± 0.07^{a}
L. rhamnosus INIA P344	–	3.84 ± 0.10^{b}	3.77 ± 0.02^{b}	3.75 ± 0.04^{b}	3.80 ± 0.07^{b}	3.63 ± 0.07^{ab}
L. rhamnosus GG	–	3.84 ± 0.11^{b}	3.79 ± 0.05^{b}	3.74 ± 0.02^{b}	3.77 ± 0.12^{b}	3.60 ± 0.06^{ab}

[a–i]Different superscript letter indicate statistically significant differences ($P < 0.01$, Tukey test). Values are mean ± SD.

Table 4
Concentration of isoflavones (μM) in fermented soy milk with selected lactobacilli and bifidobacteria over refrigerated storage.

		Daidzin	Daidzein	Genistin	Genistein
Control soy beverage	Initial*	164.81 ± 3.53^{fgh}	24.09 ± 1.14^{a}	429.66 ± 11.41^{j}	29.50 ± 0.75^{a}
	28 d†	159.26 ± 4.49^{fg}	24.25 ± 1.49^{a}	412.80 ± 7.87^{ij}	29.70 ± 0.85^{a}
B. breve INIA P734	Initial*	124.28 ± 23.00^{cd}	102.01 ± 3.87^{d}	104.60 ± 13.75^{d}	359.84 ± 40.88^{f}
	28 d†	78.30 ± 9.93^{a}	196.64 ± 10.74^{h}	36.29 ± 2.15^{ab}	435.11 ± 7.02^{g}
B. longum INIA P132	Initial*	168.56 ± 28.58^{gh}	24.7 ± 3.64^{a}	410.57 ± 32.26^{ij}	30.76 ± 0.86^{a}
	28 d†	139.38 ± 39.61^{de}	25.94 ± 2.05^{a}	410.36 ± 15.49^{ij}	31.07 ± 2.23^{a}
B. longum BB536	Initial*	177.69 ± 12.59^{h}	25.29 ± 1.38^{a}	411.96 ± 14.58^{ij}	31.61 ± 0.73^{a}
	28 d†	164.12 ± 7.71^{fgh}	25.92 ± 1.65^{a}	402.26 ± 11.03^{i}	35.13 ± 1.36^{a}
L. paracasei INIA P272	Initial*	129.11 ± 9.53^{cd}	99.52 ± 5.97^{d}	181.63 ± 8.31^{f}	267.70 ± 16.71^{d}
	28 d†	112.67 ± 32.67^{bc}	115.63 ± 18.36^{e}	134.88 ± 19.44^{e}	309.21 ± 47.08^{e}
L. rhamnosus INIA P344	Initial*	75.16 ± 5.44^{a}	193.00 ± 3.88^{h}	26.64 ± 1.89^{ab}	444.41 ± 7.76^{gh}
	28 d†	65.82 ± 11.43^{a}	226.29 ± 4.73^{i}	19.90 ± 2.21^{a}	472.79 ± 15.26^{h}
L. rhamnosus GG	Initial*	101.19 ± 7.56^{b}	152.49 ± 6.43^{f}	64.65 ± 2.23^{c}	424.53 ± 12.27^{g}
	28 d†	97.12 ± 14.90^{b}	172.44 ± 5.12^{g}	44.88 ± 3.52^{bc}	444.99 ± 15.16^{gh}

*Concentration in soy beverage fermented for 24 h. †Concentration in fermented soy beverage after refrigerated storage for 28 days.
[a–j]Different superscript letter within a column indicate statistically significant differences ($P < 0.01$, Tukey test). Values are mean ± SD.

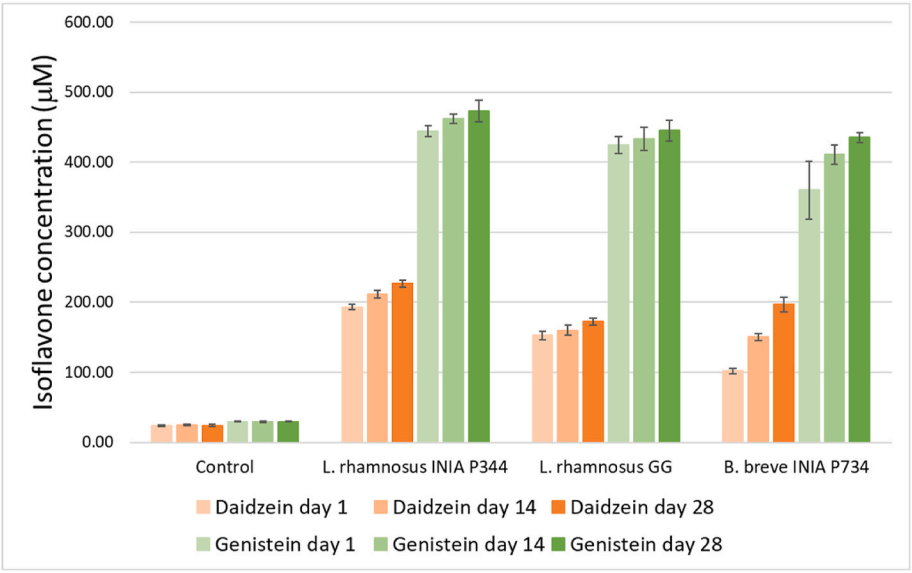

Fig. 1. Isoflavone aglycones levels during refrigerated storage of soy beverages fermented with selected *Lacticaseibacillus* and *Bifidobacterium* strains.

3.3. Antioxidant activity and phenolic compounds

Antioxidant activity showed no significant differences between the control soy beverage and those fermented with any of the bifidobacteria strains, neither after fermentation nor at any of the storage times (Table 5). On the contrary, the beverages fermented

Table 5
Antioxidant capacity as Trollox equivalens (mg/ml) of soy milk fermented with selected lactobacilli and bifidobacteria over refrigerated storage.

	Fermentation	Refrigerated storage			
	24 h	7 days	14 days	21 days	28 days
Control soy beverage	0.96 ± 0.07^{a}	0.94 ± 0.08^{a}	0.98 ± 0.06^{a}	0.95 ± 0.05^{a}	0.94 ± 0.03^{a}
B. breve INIA P734	0.85 ± 0.06^{a}	0.87 ± 0.09^{a}	0.85 ± 0.07^{a}	0.87 ± 0.05^{a}	0.84 ± 0.04^{a}
B. longum INIA P132	0.86 ± 0.03^{a}	0.89 ± 0.03^{a}	0.87 ± 0.06^{a}	0.88 ± 0.07^{a}	0.86 ± 0.06^{a}
B. longum BB536	0.98 ± 0.09^{a}	0.95 ± 0.08^{a}	0.91 ± 0.08^{a}	0.86 ± 0.03^{a}	0.82 ± 0.03^{a}
L. paracasei INIA P272	1.35 ± 0.11^{bcde}	1.34 ± 0.16^{bcde}	1.49 ± 0.13^{de}	1.51 ± 0.13^{e}	1.50 ± 0.05^{de}
L. rhamnosus INIA P344	1.26 ± 0.08^{bc}	1.27 ± 0.09^{bc}	1.21 ± 0.06^{bc}	1.30 ± 0.05^{bcd}	1.40 ± 0.12^{cde}
L. rhamnosus GG	1.21 ± 0.03^{bc}	1.19 ± 0.04^{b}	1.23 ± 0.06^{bc}	1.26 ± 0.05^{bc}	1.26 ± 0.05^{bc}

[a–e]Different superscript letter indicate statistically significant differences ($P < 0.01$, Tukey test). Values are mean ± SD.

with the lactobacilli strains showed a significant increment ($P < 0.01$) in the antioxidant activity, encompassed between 24% and 60% compared with the respective control beverages, which was maintained through the refrigerated storage.

On the other hand, only the soy beverage fermented with *B. longum* INIA P132 showed significantly ($P < 0.01$) lower levels of phenolic content than the control beverages, with 0.72 ± 0.15 versus 1.29 ± 0.09 gallic acid equivalents (mg/ml) in average respectively. The rest of the fermented beverages showed average phenolic content slightly inferior to de control but not significantly different ($P < 0.01$). The phenolic content did not vary through the refrigerated storage ($P < 0.01$) (data not shown).

4. Discussion

Isoflavones and probiotics are associated with beneficial effects on human health, and the combination of both could increase in their positive effects [30,31], including a synergistic effect [32,33]. Hence, there is an interest in the intake of these compounds for its potential benefits on health; the development of probiotic beverages enriched in bioactive isoflavones would be of great interest for the consumer.

Soy beverages represent the most important source of isoflavones. However, isoflavones are found in nature mainly in their glycosylated forms daidzin and genistin, which are not absorbed by the intestine. These glycosides must be hydrolyzed to aglycones (daidzein and genistein) to become bioavailable and physiologically active [34,35]. The fermentation of soy beverages with selected LAB and *Bifidobacterium* strains is a way to obtain a product enriched in these bioactive compounds, alongside with other isoflavone derived compounds, as well as other bioactive flavonoids [12,25,36].

An adequate concentration of the bioactive compounds and the probiotic strains should be ensured throughout the self-life of the product if a double functional effect is wanted. Therefore, the ability of lactobacilli and bifidobacteria probiotic strains to produce aglycones was evaluated, beside the survival of the strains during the refrigeration of the product.

Regarding the production of bioactive isoflavones, the two *L. rhamnosus* strains and *B. breve* INIA P734 showed a high production of bioactive isoflavones, which was maintained or even increased during storage. The evolution of isoflavone concentrations during storage has been scarcely studied and the few studies addressing it have reported the stability of the aglycones in fermented soy beverage at refrigerated temperature with a slight tendency towards degradation [25,36]. Interestingly, we observed that refrigerated storage of fermented soy beverages did not negatively affect to the levels of aglycones, on the contrary, there was a significant increase with some of the strains, which could be a consequence of the residual metabolic activity of the strains or the β-glycosidases. In this regard, *B. breve* INIA P734 registered the most outstanding transformation of glycosides to aglycones during cold storage, despite the drop in its counts, duplicating the concentration of daidzein and becoming one of the strains with higher levels of these bioactive compounds. This could be related to the distribution of β-glycosidases in bifidobacteria, which have been described to be mainly intracellular [37] and hence could increase the daidzein production after bacterial lysis.

Concerning the survival, although soy beverages have been described to be a good substrate for lactobacilli and bifidobacteria, the ability to ferment it seems to be a strain-specific trait [11,38] and can be influenced by the composition of the soy beverage [9]. The commercial soy beverage used in this work resulted in a good growth substrate for all the strains tested, which reached levels comparable to the ones described in literature for lactobacilli and bifidobacteria strains belonging to the same species [9,39–41]. Differences were observed between the lactobacilli and *Bifidobacterium* strains. Although the growth of the six strains was observed during the fermentation, the three lactobacilli strains maintained their viability, even increasing their concentration after 28 days of fermentation, whereas the refrigerated storage caused a decrease in the viable counts of bifidobacterial strains. Nevertheless, *B. breve* INIA P734 and the two *B. longum* showed good survival rates in the fermented soy beverage during the first 7 days of storage, and *B. longum* BB536 registered counts higher than 8 log units after 14 days of refrigeration. Other works have shown variability in the survival between bifidobacterial strains during refrigerated storage in fermented soy beverages [40], which could be related to a higher sensitivity of the bifidobacteria to oxygen and acid in refrigerated products [42,43].

All the fermented beverages showed pH levels below the point of gelation of soy beverage [44] and hence experienced a textural change into a soft yogurt-like curd. Here, the pH was lower in the fermented beverages with lactobacilli than with bifidobacteria strains, accordingly with other works [9,45]. The pH was generally not affected by the refrigerated storage. *L. paracasei* INIA P272 and both *L. rhamnosus* strains demonstrated good capacity for the acidification of soy beverage.

Antioxidant capacity of soy products have been linked to their content in phenolic compounds, including a higher antioxidant activity of aglycones compared with the correspondent glycosides [46,47]. However, our results showed a lack of correlation between antioxidant activity, according to the method used, and aglycone production or phenolic content of the beverages with each bacterial strain. Like this, the soy beverages fermented with lactobacilli strains showed higher antioxidant activity, while the beverage fermented with *B. breve* INIA P734, which produced similar levels of aglycones to the lactobacilli, had, on the contrary, an antioxidant activity not higher than that of the unfermented beverage. Similarly, Lee et al. [48] found no differences in the antioxidant capacity of aglycones and glycosides when tested by means of FRAP. The lack of increment in the levels of phenolics, or even decrease in the case of *B. longum* INIA 132, is in concordance with the described by Lodha et al. [49], who described also an increment in the antioxidant capacity in spite of reducing the levels of phenolics. The results obtained in our work lead us to interpret that the antioxidant capacity detected in those soy beverages fermented with lactobacilli could be related to other factors different than the phenolic or aglycone content. In this regard, it has been described that proteic compounds, such as peptides and free amino acids contribute to the antioxidant capacity [50] and that proteolytic activity and low pH are correlated with higher antioxidant capacities in soy beverages fermented with lactobacilli [51]. Hence, the characteristic high proteolytic activity of lactic acid bacteria [52], and the lower pH would be contributing to a higher antioxidant capacity after fermentation with the lactobacilli strains and throughout the refrigerated storage of the fermented beverages.

Taken together, these results demonstrated that fermented soy beverages are very promising products as a vehicle for probiotic bacteria, as well as for bioactive isoflavones. The selection of a functional starter strain for the production of an enriched probiotic beverage must take into account a good growth during fermentation and the ability of the strain to produce aglycones. Moreover, the stability of those aglycones and the levels of live microorganisms during storage are crucial to obtain a product with double functional benefit as a source of aglycones and probiotics.

5. Conclusion

Strains with probiotic traits, such as *L. rhamnosus* GG and *L. rhamnosus* INIA P344, produced high levels of aglycones while maintaining their viability during the refrigerated storage. Refrigerated storage over 28 days did not reduce the concentrations of daidzein and genistein obtained in soy beverage fermented with probiotic strains. Together with their ability to acidify the fermented soy beverages, these characteristics make these strains interesting starters to obtain fermented soy beverages that would merge the health benefits of both bioactive isoflavone aglycones and probiotic strains. The described probiotic and technological properties of *L. rhamnosus* GG and *L. rhamnosus* INIA P344 make them attractive candidates as starters that would allow the development of a fermented beverage with a potential double health interest for consumers.

Funding source

This work was supported by the projects PID2020-11960RB-I00 from the Spanish Ministry of Science and Innovation. A.R.d.l.B. is recipient of a Pre-doctoral contract (PRE2018-086293).

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