Article

Optimization of the Bioactivation of Isoflavones in Soymilk by Lactic Acid Bacteria

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Abstract: Soybeans and soy-based products contain isoflavones which can be used for nutraceutical and medical applications. In soybeans and in unfermented soy foods, isoflavones are normally present as glycosides. Isoflavone glycosides can be enzymatically converted to isoflavone aglycones, thus releasing the sugar molecule. The effective absorption of isoflavones in humans requires the bioconversion of isoflavone glycosides to isoflavone aglycones through the activity of the enzyme β-glucosidase. The objective was to assess the capacity of 42 bacterial strains (belonging to *Lactobacillus*, *Streptococcus* and *Enterococcus*) to produce β-glucosidase activity. The strain that showed the highest β-glucosidase activity (*Lactobacillus plantarum* 128/2) was then used for the optimization of the bioconversion of genistin and daidzin present in commercial soymilk to their aglycone forms genistein and daidzein. The contribution of process parameters (temperature, inoculum size, time) to the efficiency of such bioactivation was tested. *Lactobacillus plantarum* 128/2 was able to completely bioactivate soymilk isoflavones under the following conditions: 25 °C temperature, 2% inoculum size and 48 h process time. These results confirm the suitability of lactic acid bacteria for the bioactivation of isoflavones present in soymilk and provide an interesting candidate (*L. plantarum* 182/2) for food industries to perform this transformation.

Keywords: β-glucosidase; isoflavone glycosides; isoflavone aglycones; nutraceuticals; phytoestrogens

1. Introduction

Isoflavones are a group of flavonoids similar in structure and biological activity to endogenous 17β-estradiol [1]. Due to this structural similarity to the human female hormone 17β-estradiol, isoflavones can bind to estrogen receptors and show estrogen-like activities. Isoflavones are present in large quantities in legumes [2] and have attracted considerable interest in the last decades and years due to their possible beneficial effects for a variety of human diseases and their potential for the development of functional foods. In fact, the consumption of isoflavones has long been linked to a variety of health benefits such as, for instance, lower risk of cardiovascular disease and breast and prostate cancer, attenuated menopausal symptoms, prevention of bone loss, osteoporosis, etc. [1,3–5]. Nonetheless, isoflavones have also been reported as potential endocrine disruptors and, hence, with the capacity to cause adverse effects on human health [5,6]. Actually, there are growing concerns about an excessive exposure of humans to isoflavones owing to their estrogen-like properties [7,8]. At this time, there seems to be a consensus regarding the need for more studies on this issue, in order to confirm, rule out or put into context the potential health benefits (possibly, modest effects) linked to the consumption of soy isoflavones [2,9].

Soybeans (*Glycine max* L.) and soy-based foods are the major source of isoflavones in the human diet. In Asia, soybeans are consumed without much processing, whereas in western countries soy-based products are consumed in highly processed forms [5]. It has been reported [5,10] that processed soy products may not have the same health benefits as the unprocessed soy foods. In any case, in an attempt to benefit from the abovementioned potential positive effects of isoflavones on human health, the consumption of soymilk (as well as its by-products) and foodstuffs enriched with isoflavones is often encouraged.

In soybeans and in unfermented soy foods, isoflavones are usually present in a conjugated form (linked to sugars) [3]. In fact, soy isoflavones can exist as glycosides (conjugated to sugars) or as aglycones (hydrolysates of isoflavone glycosides). In the human gut, isoflavone glycosides are hydrolyzed to their corresponding aglycone forms (by bacteria that colonize the human intestine) which are then easily transported across intestinal epithelial cells [11]. Unlike aglycones, glycosides cannot be absorbed owing to their higher hydrophilicity and higher molecular mass and, therefore, they can be metabolized only when hydrolyzed [5,12]. The effective absorption of isoflavones requires the bioconversion of glycosides to aglycones through the activity of the enzyme β-glucosidase produced by gut bacteria [3]. Then, the absorption of isoflavones in humans can differ among populations due to factors such as the composition of the intestinal microbiota, dietary habits, ethnic background, etc. [13,14]. Bacterial species of Bacteroides, Bifidobacteria and Lactobacilli have been reported to show the highest levels of β-glucosidase activity [15]. Interestingly, it has been observed [16] that the chronic ingestion of soy can lead to an increase of intestinal β-glucosidase activity. However, the efficiency of the enzymatic bioconversion of isoflavone glycosides to isoflavone aglycones depends on many factors, such as the microbial species, inoculum size, process time, temperature, etc. For instance, temperature is well-known to affect the growth of microorganisms and, hence, the activity of the enzymes produced by them.

Genistin (4′,5,7-trihydroxyisoflavone), daidzin (4′,7-dihydroxyisoflavone) and glycitin (7,4′-dihydroxy-6-methoxy-isoflavone) are the most relevant soy isoflavone glycosides [15]. Their corresponding isoflavone aglycones are termed genistein, daidzein and glycitein. In the conjugated form, soy isoflavones can be present as glucosides, acetylglucosides (6″-O-acetyldaidzin, 6″-Oacetylgenistin, 6″-O-acetylglycycitin) and malonylglucosides (6″-O-malonyldaidzin, 6″-O-malonylgenistin, 6″-O-malonylglycitin) [17]. Thus, soybeans contain three types of isoflavones in four chemical forms: glucosides (daidzin, genistin, glycitin), acetylglucosides (acetyldaidzin, acetylgenistin, acetylglycitin), malonylglucosides (malonyldaidzin, malonylgenistin, malonylglycitin) and aglycones (daidzein, genistein and glycitein), resulting in a total of 12 different isoflavones [2]. The type and content of these isomers in foods varies, among other aspects, depending on the plant part from which they are derived and the method by which they are processed [10,18,19]. Genistein, daidzein and glycitein comprise approximately 50, 40 and 10% of soy isoflavone content, respectively [4,20].

Some relevant non-fermented soy-based food products are: soymilk, tofu, yuba, soybean sprouts, okara, roasted soybeans, soy nuts, soy flour, etc. Similarly, important fermented soy-based food products are soy paste, soy sauce, tempeh, natto, soy nuggets, sufu, etc. [2]. Fermentation of soybeans is known to alter their content of isoflavones: for instance, the content of genistein in fermented soybean products is higher than in unfermented soybeans and soybean products such as soymilk and tofu [3]. Indeed, although the fermentation of soy can significantly reduce the total content of isoflavonoids [11], their bioavailability is normally higher in fermented products [3,11].

Some authors [8] have studied the content of isoflavones in soy agricultural waste. Apart from the possibility of obtaining a high-added value product from such waste, their management and processing could also help mitigate undesirable impacts to the environment, since the soy waste left in the agricultural field might be leached by rainwater causing environmental contamination with phytoestrogens [8]. This strategy is in accordance with the circular bioeconomy paradigm (the term bioeconomy refers to

the production of renewable biological resources and their transformation into nutrients, bio-based products, and bioenergy). Actually, the production of pharmaceuticals and nutraceuticals (such as those derived from soy isoflavones) is one of the main goals of many bioeconomy and bio-based economy studies. Nowadays, the development and implementation of circular bioeconomy and bio-based initiatives for creating value-added products is highly encouraged.

The objectives of this study were: (i) to examine the capacity of 42 bacterial strains (belonging to *Lactobacillus*, *Streptococcus* and *Enterococcus*) to produce β-glucosidase activity, (ii) to assess the isoflavone content of different soy-based products, and (iii) to test the capacity of the strain that showed the highest β-glucosidase activity to drive the bioactivation of genistin and daidzin present in commercial soymilk to their corresponding aglycone forms genistein and daidzein. The contribution of some process parameters (temperature, inoculum size, and time) to the efficiency of such bioactivation was tested.

2. Materials and Methods

2.1. Materials

Soybeans (*Glycine max* L.) and commercial soy-based products (three soymilks called here, for confidentiality purposes, No. 1, No. 2, and No. 3, and two soy-based yoghurts called here No. 4 and No. 5) were purchased from a local market. For comparison purposes, a homemade soy milk was also prepared as follows: the soybeans were washed and soaked overnight in distilled water (1: 6 *w/v* ratio of soybeans to water). The soaked soybeans were then cooked at 90 °C for 20 min in a 2 L beaker. After decanting the liquid phase, soybeans were crumbled in a blender (MX050 Dynamic, Mortagne-sur-Sèvre, France) and the resultant slurry was filtered with double layers of cheesecloth to remove solid particles. Afterwards, the liquid was transferred into glass bottles (Duran Group, Wertheim/Main, Germany) and sterilized by autoclaving at 121 °C for 15 min. The resulting homemade soymilk was cooled and, finally, stored at 4 °C until use.

Reagents for the preparation of bacterial culture media (Man Rogosa Sharpe-MRS broth and Brain Heart Infusion-BHI broth) were obtained from Sigma-Aldrich (Steinheim, Germany). For the determination of β-glucosidase activity, p-nitrophenyl β-D-glucopyranoside (pNPG) and p-nitrophenol were purchased from Sigma-Aldrich (Steinheim, Germany). Isoflavone glycosides (daidzin and genistin) and corresponding aglycones (daidzein and genistein) were purchased from Sigma-Aldrich (Steinheim, Germany). Isoflavones were diluted to 1000 mg L^{-1} solutions using dimethyl sulfoxide (DMSO) (99.9%, LabScan, Dublin, Ireland). Acetic acid (99.7%) and acetonitrile (99.9%) were obtained from Panreac (Barcelona, Spain). Methanol (MeOH), ethyl acetate and n-hexane (HPLC quality) were obtained from LabScan (Dublin, Ireland). Disodium hydrogen phosphate (100%) (Panreac, Barcelona) and citric acid (99.5%) (Sigma-Aldrich, Steinheim) were used for the preparation of the required buffer solution (5 mmol, pH 6.8).

Finally, all bacterial strains studied here were kindly provided by the Dairy Institute of Asturias (CSIC, Villaviciosa, Spain).

2.2. Preparation of Bacterial Inocula

The first objective of this study was to assess the capacity of 42 bacterial strains (belonging to *Lactobacillus*, *Streptococcus* and *Enterococcus*) to produce β-glucosidase activity. To this purpose, 27 *Lactobacillus* strains were maintained in MRS agar stabs at 4 °C (Table 1). After two successive transfers in MRS broth at 37 °C for 12–15 h, *Lactobacillus* cells in exponential phase were re-inoculated into MRS broth and grown at 37 °C for 16 h. These bacterial cultures were used as inoculum for the below-mentioned tests on β-glucosidase activity.

Table 1. Lactobacillus, Streptococcus and Enterococcus strains used in this study.

	Strain	Culture Medium
1	*L. acidophillus*	MRS [a]
2	*L. acidophillus* 4353	MRS [a]
3	*L. brevis* 3810	MRS [a]
4	*L. brevis* 3811	MRS [a]
5	*L. brevis* 3824	MRS [a]
6	*L. brevis* 4121	MRS [a]
7	*L. brevis* 5354	MRS [a]
8	*L. brevis* C310	MRS [a]
9	*L. casei*	MRS [a]
10	*L. delbruecki* 11842	MRS [a]
11	*L. delbruecki* 20074	MRS [a]
12	*L. fermentum* 4339	MRS [a]
13	*L. fermentum* PND4	MRS [a]
14	*L. gasseri* 98552	MRS [a]
15	*L. gasseri* H633	MRS [a]
16	*L. leichmanii*	MRS [a]
17	*L. paraplantarum* CuB7	MRS [a]
18	*L. plantarum* 128/2	MRS [a]
19	*L. plantarum* MCMB 8826	MRS [a]
20	*L. plantarum* LC441	MRS [a]
21	*L. plantarum* LPC 01 nic 100	MRS [a]
22	*L. rhamnosus* CR1	MRS [a]
23	*L. reuteri* 213	MRS [a]
24	*L. reuteri* PND1	MRS [a]
25	*L. reuteri* PND3	MRS [a]
26	*L. reuteri* PND7	MRS [a]
27	*L. helveticus*	MRS [a]
28	*S. thermophillus* CN RZ 1066	BHI [b]
29	*S. thermophillus* CN RZ 1205	BHI [b]
30	*S. thermophillus* CMD9	BHI [b]
31	*S. thermophillus* 5	BHI [b]
32	*S. thermophillus* 6.10.2	BHI [b]
33	*S. thermophillus* 6.6.1	BHI [b]
34	*S. thermophillus* 3.2.4	BHI [b]
35	*S. thermophillus* 111.2	BHI [b]
36	*E. faecium* CM17 HFS14	BHI [b]
37	*E. faecium* CM17 HFS7	BHI [b]
38	*E. duraus* C6	BHI [b]
39	*E. faecalis*	BHI [b]
40	*E. faecium* CM17 C39	BHI [b]
41	*E. faecium* CM17 HFS11	BHI [b]
42	*E. duraus* CELT 441	BHI [b]

[a] MRS: Man Rogosa Sharpe, [b] BHI: Brain Heart Infusion.

Moreover, eight strains of *Streptococcus thermophillus* and seven strains of *Enterococcus* were maintained in BHI agar stabs at 4 °C (Table 1). After two successive transfers in BHI broth at 37 °C for 24 h, bacterial cells in exponential phase were re-inoculated into BHI broth and grown at 37 °C for 24 h. Similarly, these bacterial cultures were used as inoculum for the below-mentioned tests on β-glucosidase activity.

2.3. β-Glucosidase Activity

The β-glucosidase activity of all the bacterial strains was quantified as described in Di Cagno et al. [21]. Briefly, the reaction mixture contained 500 μL of 0.5 mM pNPG in 5 mM phosphate/citrate buffer (pH 6.8), and 500 μL of the corresponding bacterial cell suspension at an optical density (OD_{600}) of 1.0. The mixture was incubated at 40 °C for 30 min. Then, the reaction was stopped by the addition of 500 μL of an ice-cold

2 M sodium carbonate solution. The mixture was then centrifuged (14,000 rpm, 5 min) (Beckman Coulter Avanti J-26 XP, Fullerton, California, USA) and the amount of released p-nitrophenol was spectrophotometrically (Shimadzu UV-2401PC, Tokyo, Japan) determined at 400 nm. One unit (U) of enzyme activity was defined as the amount of β-glucosidase that releases 1 μmol of p-nitrophenol per milliliter and per min, under the assayed conditions.

2.4. Quantification of Isoflavones

Once the 42 bacterial strains had been tested for their capacity to produce β-glucosidase activity, isoflavone content was determined in the five abovementioned commercial soy-based products (three milks, two yoghurts), as well as in the homemade soymilk. The extraction of isoflavones from these food products was carried out as described by Otieno et al. [22] and Otieno and Shah [23]. Briefly, soy-based products were diluted fifteen times with MeOH. Then, 1 mL of this solution was heated in an oven at 65 °C for 30 min. The insoluble residue was separated by centrifugation (3700 rpm, 5 min, 4 °C), while the supernatant was evaporated to dryness under a gentle stream of nitrogen in a Turbovap LV Evaporator (Zymark, Hopkiton, MA, USA). The concentrated extract was re-dissolved in 1 mL of the mobile phase: 1% (*v/v*) acetic acid in water and 1% (*v/v*) acetic acid in MeOH at a ratio of 85:15 (*v/v*). Finally, the extract was filtered with a 0.45 μm syringe filter (Millipore®, Bedford, MA, USA) and introduced into a HPLC vial.

Isoflavone content was determined by HPLC (Agilent Technologies, Avondale, PA, USA) using an Agilent DAD detector and an Agilent autosampler. Two microliters of the above-mentioned extract were injected in a ZorbaxEclipse XDB-C18 (150 × 4.6 mm, 5 μm) column. The samples were eluted at 1 mL min^{-1} with a linear gradient of 1% (*v/v*) acetic acid in water (A) and 1% (*v/v*) acetic acid in MeOH (B). The elution gradient (a four-step linear gradient) was started immediately after injection. The elution gradient was as follows: 15% B was maintained for 1 min, later increased to 40% B for 6 min, and then to 60% B for 30 s, where it was held constant for 2.5 min. At the end of the run, the column was re-equilibrated with 15% B for 2 min before the next run. The DAD was operated between 200 and 800 nm, and chromatograms for quantitative analysis were extracted at 254 nm. Isoflavones were identified by their retention time. Multi-wavelength UV spectrums were compared with those of standards. Calibration curves for genistin, daizin, genistein and daizein were obtained using concentrations in the range of 0.02–20.00 μg mL^{-1}.

2.5. Bioactivation of Isoflavones in Soymilk

After testing the 42 bacterial strains, it was observed that *Lactobacillus plantarum* 128/2 showed the highest β-glucosidase activity and, in consequence, it was selected for the study of the optimization of the bioactivation of genistin and daidzin present in soymilk to their corresponding aglycone forms (genistein and daizein). To this purpose, the commercial soymilk No. 1 was used, as it presented the highest isoflavone content (see below).

Initially, *L. plantarum* 128/2 cells were propagated twice in 10 mL of MSR broth at 37 °C for 12–15 h, to obtain an OD$_{600}$ of 1.0. Then, the commercial soymilk No. 1 was inoculated with this bacterial culture (2% *v/v* = inoculum size) and kept at 25 °C and 37 °C for 24 h (to evaluate the effect of growth temperature on the abovementioned bioactivation). Subsequently, samples from the inoculated soymilk were aseptically withdrawn and immediately cooled on ice to then measure isoflavone content.

Similarly, the effects of inoculum size (2% vs. 10%) and process time (24 h vs. 48 h) were also tested, in order to optimize process conditions. All tests were run in triplicate.

2.6. Statistical Analysis

Results are presented as mean values ± standard deviations. One-way analysis of variance (ANOVA) was used for comparisons among β-glucosidase activities. A *p*-value < 0.05 was considered as statistically significant.

3. Results and Discussion

3.1. β-Glucosidase Activity

Nowadays, the consumption of soy-based food products is often being encouraged in many western countries where, unlike in many Asian countries, soy has traditionally not been an integral part of the regular diet. The consumption of these products has been linked to a variety of potential positive health effects (e.g., reduction in incidence and severity of cardiovascular diseases, breast and prostate cancer, menopausal symptoms, etc.) [1,3–5], but some potential adverse health effects have also been reported [5,6,8,13]. In consequence, at the moment, a regular recommendation, for precautionary reasons, is to consume moderate amounts of traditionally prepared, minimally processed soy foods [2], in an attempt to benefit from the positive properties of soy isoflavones while minimizing their potential adverse health effects [24], until more extensive rigorous studies on this topic are performed.

One of the most common soy-based products consumed in western countries is soymilk. As previously mentioned, soy isoflavone composition appears a key determinant of isoflavone effects. In this respect, the absorption of soy isoflavone in humans highly depends on the conversion of isoflavone glycosides to their corresponding aglycones through the activity of the enzyme β-glucosidase produced by gut bacteria [3,15]. In addition, thanks to the activity of the gut microbiota, daidzein may later be metabolized to form equol (7-hydroxy-3-(4′-hydroxyphenyl)-chroman) and O-desmethylangolensin [25]. The ability to produce equol (not all soy consumers produced it efficiently) [12] appears determinant for the claimed beneficial effects of isoflavones [26,27]. Finally, in their studies with *Lactobacillus* GG, *Lactobacillus acidophilus* and *Bifidobacterium bifidus*, Larkin et al. [28] suggested that β-glucuronidase activity might be more important than β-glucosidase activity for isoflavone bioavailability.

Here, 42 bacterial strains (twenty-seven *Lactobacillus*, eight *Streptococcus* and seven *Enterococcus*) were tested for their β-glucosidase activity under optimal growth conditions (Figures 1 and 2). Regarding *Lactobacillus* strains (Figure 1), *L. plantarum* 128/2 showed the highest levels of β-glucosidase activity (No. 18 = 43 ± 15 mU mL^{-1}), followed by *L. plantarum* LPC 01 nic 100 (No. 21 = 41.0 ± 0.3 mU mL^{-1}) and *L. plantarum* MCMB 8826 (No. 19 = 28 ± 10 mU mL^{-1}). The level of β-glucosidase activity shown by *L. plantarum* 128/2 and *L. plantarum* LPC 01 nic 100 was significantly ($p < 0.05$) higher, compared to all the other strains (Figure 1).
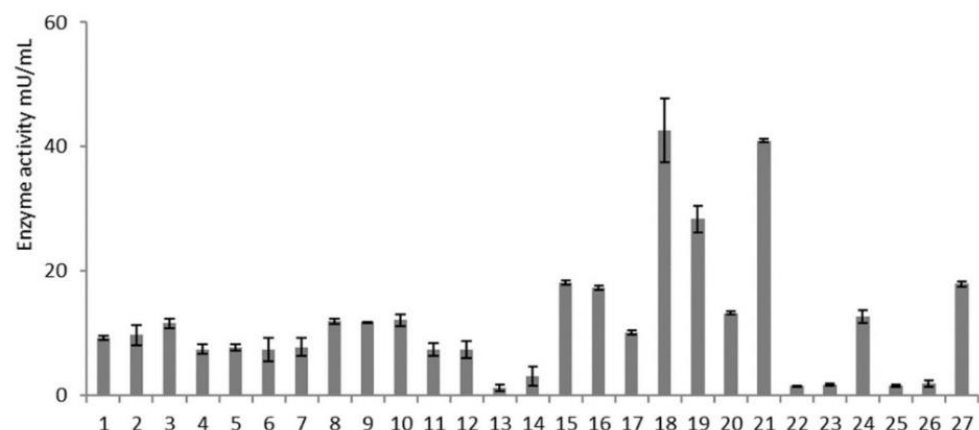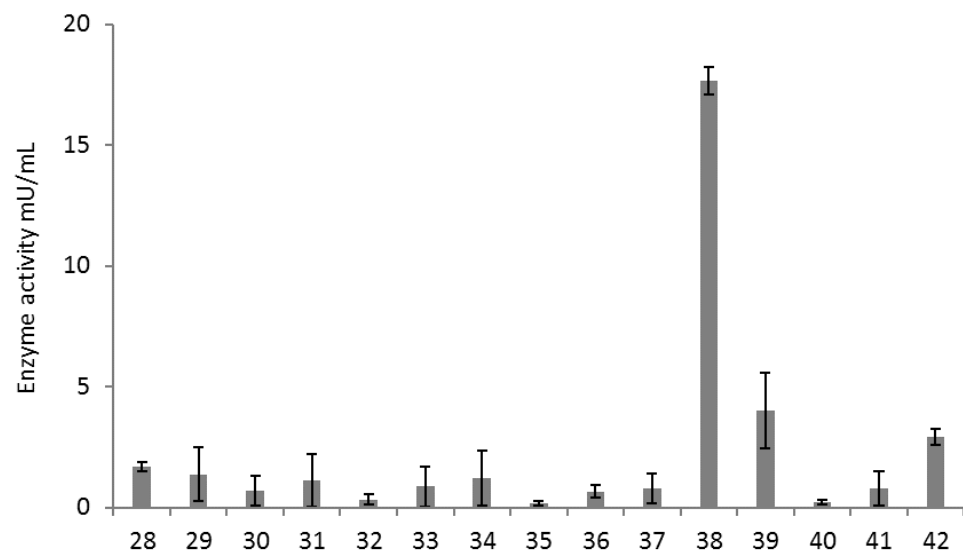

Figure 1. β-glucosidase activity of *Lactobacillus* strains.

Figure 2. β-glucosidase activity of *Streptococcus* and *Enterococcus* strains.

Streptococcus thermophillus strains showed very low levels of β-glucosidase activity (Figure 2). Finally, among the seven *Enterococcus* strains tested here, *E. duraus* C6 clearly showed the highest level of β-glucosidase activity (No. 38 = 17.7 ± 0.3 mU mL^{-1}) (Figure 2). These results agree with those reported by other authors [15,22,29,30] where *Lactobacillus* cultures showed higher values of β-glucosidase activity, compared to other intestinal bacteria. In addition, Yuksekdag et al. [31] screened 54 strains (belonging to *Lactobacillus*, *Bifidobacterium* and *Propionibacterium*) for β-glucosidase activity, finding values from 0.250 to 3.000 U mg^{-1}. *Propionibacterium* strains showed lower values of β-glucosidase activity than *Lactobacillus* and *Bifidobacterium* strains (highest values were observed for *Lactobacillus*). Similar results were observed by Marazza et al. [32]. The production of soy isoflavone aglycones from isoflavone glycosides has also been studied with β-glucosidase isolated from fungi (i.e., the filamentous fungi *Aspergillus oryzae*) [17].

Lactobacillus plantarum is a bacterial species of great interest as it possesses relevant enzymatic activities to obtain simpler and more biologically active compounds via the enzymatic transformation of phenolic compounds [33]. Importantly, the adaptative behavior of *L. plantarum* under phenolics-induced stress modulates several traits beneficial for its gastrointestinal survival [33]. These results emphasize the importance of species and strain selection prior to the use of bacterial strains for the bioactivation of isoflavones.

In light of these results, *L. plantarum* 128/2 was selected for the study of the optimization of the bioactivation of genistin and daidzin present in soymilk (commercial soymilk No. 1) to their corresponding aglycone forms (genistein and daizein).

3.2. Bioactivation of Isoflavones in Soymilk

As described above, five commercial soy-based products (three milks, two yoghurts), as well as the homemade soymilk, were analyzed regarding their content of isoflavones. Commercial soymilk No. 1 clearly showed the highest content of isoflavone glycosides (Figure 3). On the other hand, the homemade soymilk showed the highest content of isoflavone aglycones. In any event, the content of isoflavones (both glycosides and aglycones) varied considerably among the studied food products (Figure 3), possibly due to differences in the content of isoflavones initially present in the raw material (soybeans) as well as the specific industrial process conditions used to manufacture these food products (milks and yoghurts). The five commercial soy-based products had a much higher content of isoflavone glycosides (genistin and daidzin) than of isoflavone aglycones (genistein and daidzein), suggesting their inadequacy for easy adsorption. By contrast, the homemade soy milk had a much higher content of isoflavone aglycones than of isoflavone glycosides (Figure 3). The total amount of isoflavones in soymilk products has been reported to vary

considerably among the different commercial products and even among different lots of the same product [34]. In their study on the quantification of isoflavones in soymilk and tofu, Prabhakaran et al. [35] observed higher concentrations of isoflavones in soy foods containing higher levels of protein content, compared to those with lower protein content. Total isoflavone content has been reported to vary by up to five-fold among different commercial soymilks, with whole soybean milks having significantly higher isoflavone levels than those made from soy protein isolates (63.6 $\pm$ 21.9 mg L^{-1} vs. 30.2 $\pm$ 5.8 mg L^{-1}) [36]. Likewise, the ratio of genistein to daidzein was higher in isolated soy protein-based *versus* "whole bean" soymilks (2.72 $\pm$ 0.24 vs. 1.62 $\pm$ 0.47, respectively, $p < 0.0001$) [36]. In any event, many studies have reported considerable variations in the content of soybean isoflavones according to the conditions under which they are grown [37] or stored [38], the maturity of the soybean [39], the specific strain of soybean, the geographical locations of harvest [37,40,41], etc., pointing out to the great difficulty of producing a whole bean soymilk with consistent isoflavone levels [36].
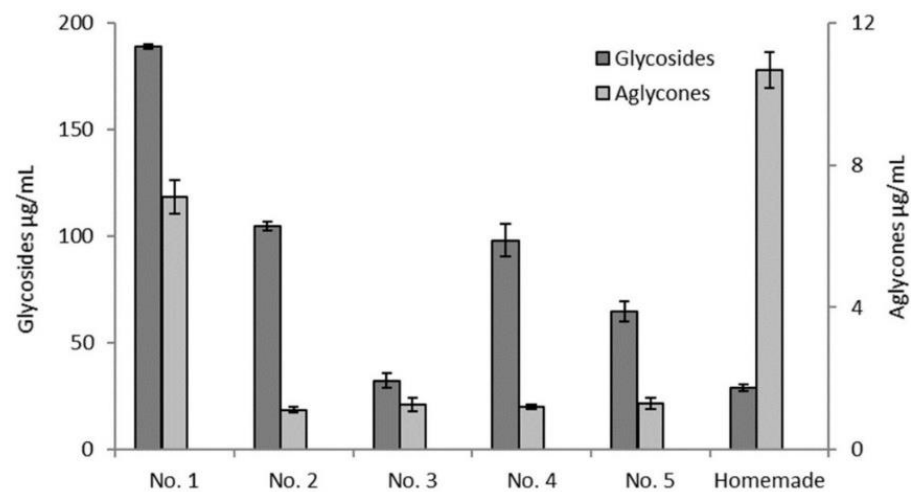

Figure 3. Isoflavone contents in soy-based food products.

According to the obtained results, commercial soymilk No. 1 was selected for the study of the capacity of *L. plantarum* 128/2 to bioactivate soy isoflavone glycosides (genistin, daidzin) to their corresponding aglycone forms (genistein, daizein). Although there is controversy on this matter and contradictory findings [3,14,42–45], isoflavone aglycones appear to be absorbed faster than isoflavone glycosides, and, in consequence, the aim of this work was to optimize the bioactivation of isoflavones in soymilk using lactic acid bacteria. In particular, as described above, for the optimization of this isoflavone bioactivation process with *L. plantarum* 128/2, two temperatures (25 °C vs. 37 °C), two inoculum sizes (2% vs. 10%) and two process times (24 h vs. 48 h) were compared.

Figure 4 shows the effect of temperature (25 °C vs. 37 °C) on the bioactivation of the isoflavone glycosides present in commercial soymilk No. 1, thanks to the β-glucosidase activity of *L. plantarum* 128/2, at an inoculum size of 2% and a process time of 24 h. The bioactivation process (conversion of genistin and daidzin to genistein and daidzein) was highly effective (though not complete) at both temperatures (Figure 4). At all times, the content of genistin was higher compared to daidzin. Concerning aglycones, values of genistein and daidzein contents were rather similar. In any case, the bioactivation was somewhat more efficient at 37 °C vs. 25 °C. Actually, the point at which the lines showing the decrease of glycoside contents intersect with the lines representing the increasing values of aglycone contents (i.e., the equilibrium point) occurred at an earlier time at 37 °C vs. 25 °C. Nonetheless, the difference between both temperatures in terms of bioactivation efficiency was rather small, which suggests the possibility of opting for 25 °C, in order to save energy and money. In any case, a complete conversion of isoflavone glycosides to isoflavone aglycones was achieved neither at 37 °C nor at 25 °C.
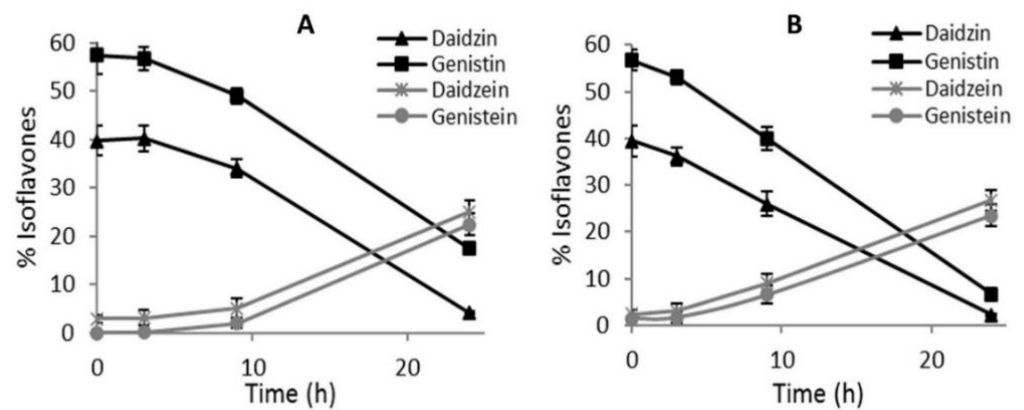
Figure 4. Effect of temperature on isoflavone bioactivation by L. *plantarum* 128/2. (**A**) 25 °C. (**B**) 37 °C.

However, in an attempt to enhance the process efficiency at 25 °C, the effect of inoculum dose at this lower temperature (Figure 5) was studied, hoping to obtain higher bioactivation rates by increasing the inoculum dose from 2 to 10%. Regrettably, increasing the dose of inoculum did not result in a noticeable improvement of bioactivation rates after 24 h. The bioconversion process did start earlier at 10% inoculum dose, compared to 2%, but as the process developed, the values became rather similar. From the results shown in Figures 4 and 5, a temperature of 25 °C and an inoculum dose of 2% were selected for the bioactivation of isoflavones in soymilk by the β-glucosidase activity of *L. plantarum* 128/2.
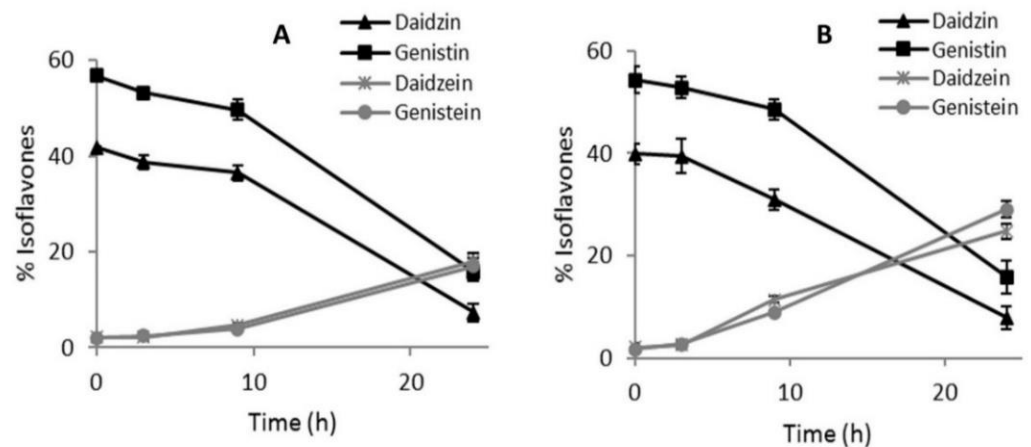

Figure 5. Effect of inoculum dose on isoflavone bioactivation by L. *plantarum* 128/2. (**A**) 2%. (**B**) 10%.

Nonetheless, the bioactivation of isoflavone glycosides to isoflavone aglycones was incomplete under all the studied experimental conditions (Figures 4 and 5). For that reason, it was decided to increase process time from 24 h to 48 h (Figure 6), using a temperature of 25 °C and an inoculum dose of 2%. At this longer process time, the bioactivation of the isoflavones (bioconversion of glycosides to aglycones) was complete. In any case, even under these more optimal conditions, glycosides represented almost 100% of isoflavones, while aglycones represented at most 65% of isoflavones (even when glycosides were completely bioconverted). This is probably due to the production of other compounds (e.g., malonylglucosides, acetylglucosides) [22].

Many *Lactobacillus* strains have previously been tested for their capacity to transform isoflavone glycosides to aglycones in soymilk under different process conditions. For instance, Hati et al. [46] fermented soymilk with six *Lactobacillus* strains (*L. rhamnosus* C6 and C2, *L. rhamnosus* NCDC19 and NCDC24, *L. casei* NCDC17 and NCDC297) at 37 °C for 12 h. The highest β-glucosidase activity and isoflavone bioconversion was achieved with *L. rhamnosus* C6 [46]. In a similar study to ours [21], one hundred and three strains of

lactic acid bacteria were assayed for β-glucosidase activity, finding out that *L. plantarum* DPPMA24W and DPPMASL33, *L. fermentum* DPPMA114, and *L. rhamnosus* DPPMAAZ1 had the highest activity values. These three strains were selected as mixed starter to ferment soymilk made with organically farmed soybeans, and, after 96 h of fermentation, the soymilk contained 57.0 μM daidzein, 140.3 μM genistein, 20.4 μM glycitein, and 37.3 μM equol. Similarly, five *Lactobacillus* strains (*L. acidophilus* B4496, *L. bulgaricus* CFR2028, *L. casei* B1922, *L. plantarum* B4495, *L. fermentum* B4655) were used for fermented soymilk isoflavone bioactivation at 37 °C for 24 h and 48 h, in combination with the yeast *Saccharomyces boulardii*, achieving very effective rates of bioactivation [47]. The novelty of such work was that, since lactic acid bacteria often present poor survival in, for instance, yoghurt (due to low pH and low acid tolerance), yeast can utilize the organic acids produced during fermentation thereby increasing the pH of the environment and, hence, the viability of the *Lactobacillus* strains. In another study [48], soymilk was fermented with *Streptococcus infantarius* 12 and/or *Weissella* sp. 4 for 12 h at 37 °C, observing that the sharp increase in β-glucosidase activity corresponded with a rapid decrease in isoflavone glycosides and an increase in the aglycone forms. Marazza et al. [49] observed a high hydrolysis degree of soymilk isoflavone glycosides (81.2%), with *Bifidobacterium longum* CRL849, after 24 h at 37 °C. In the same way, five strains of bifidobacteria were screened for β-glucosidase activity: *Bifidobacterium animalis*, *B. longum*-a, and *B. pseudolongum* caused hydrolysis of isoflavone malonyl-, acetyl- and β-glycosides to form aglycones, and the transformed daidzein to equol in soymilk [49].
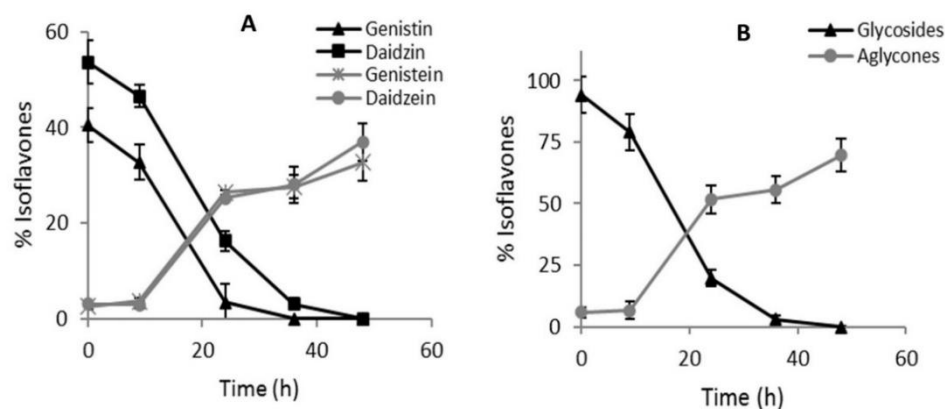
Figure 6. Effect of *Lactobacillus plantarum* 128/2 on the (**A**) bioactivation rate (%) of daidzin and genistin to daidzein and genistein after 48 h; and (**B**) bioactivation rate (%) of isoflavone glycosides to isoflavone aglycones after 48 h.

Finally, several treatments have been studied in an attempt to optimize the bioactivation of soy isoflavones. For instance, Ewe et al. [50] evaluated the effects of ultrasounds on *Lactobacillus fermentum* BT8633 growth and isoflavone bioconversion activity in soymilk, finding out that the growth of ultrasonicated cells increased by 3.2–9.1%, compared to the control. This increased growth was associated with enhanced intracellular and extracellular (8.4–17.0% and 16.7–49.2%, respectively) β-glucosidase activity, leading to increased bioconversion of isoflavones glycosides to aglycones, possibly due to the reversible permeabilized membrane of ultrasonicated cells that facilitated the transport of molecules across the membrane.

Then, it was concluded that *L. plantarum* 128/2, thanks to its great capacity to produce β-glucosidase activity, was able to completely bioactivate soymilk isoflavones under the following process conditions: 25 °C temperature, 2% inoculum dose, and 48 h process time. These process conditions could most likely still be improved (e.g., by testing a process time between 24 h and 48 h, an inoculum dose between 2% and 10%, etc.). In this study, *L. plantarum* 128/2 has shown its great potential for the bioactivation of isoflavones in soymilk, in order to improve its nutritional value.

4. Conclusions

The 42 bacterial strains studied here showed varying levels of β-glucosidase activity, capable of driving the bioactivation of isoflavone glycosides to their corresponding isoflavone aglycones. In any case, *Lactobacillus plantarum* 128/2 has shown its remarkable capacity to produce β-glucosidase and, then, to stimulate the abovementioned bioactivation of isoflavones in soymilk. Actually, *L. plantarum* 128/2, was able to completely bioactivate soymilk isoflavones under the following process conditions: 25 °C temperature, 2% inoculum dose, and 48 h process time. These results confirm the suitability of lactic acid bacteria for the bioactivation of isoflavones present in soymilk.

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