Production of *O*-desmethylangolensin, tetrahydrodaidzein, 6'-hydroxy-*O*-desmethylangolensin and 2-(4-hydroxyphenyl)-propionic acid in fermented soy beverage by lactic acid bacteria and *Bifidobacterium* strains

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ARTICLE INFO

Keywords:
Soy beverage
Bioactive isoflavones
Lactic acid bacteria
Bifidobacterium
O-desmethylangolensin
2-(4-hydroxyphenyl)-propionic acid

ABSTRACT

Isoflavones intake is associated with health benefits. The metabolism of isoflavones by bacteria plays a key role in their biotransformation. Therefore, commercial soy drink was fermented by 11 lactic acid bacteria (LAB) and 9 bifidobacteria strains. The majority of the strains showed deglycosylation of the isoflavone glycosides present in soy drink and appearance of the aglycones daidzein, genistein and glycitein. Moreover, we observed the further transformation of daidzein into *O*-desmethylangolensin (*O*-DMA) and tetrahydrodaidzein, alongside with dihydrodaidzein (DHD) and a putative isomer of DHD. On the other hand, genistein was transformed by nearly all strains into 6-hydroxy-*O*-desmethylangolensin (6-hydroxy-*O*-DMA), but no dihydrogenistein production was registered. A high concentration of 2-(4-hydroxyphenyl)-propionic acid was observed, suggesting the degradation of *O*-DMA and 6-hydroxy-*O*-DMA. The potential of LAB and *Bifidobacterium* strains to produce functional soy drink enriched with bioactive isoflavones is demonstrated in this work.

1. Introduction

Isoflavones are polyphenols biologically active, present in various plants, particularly in soybean germ (Vitale, Piazza, Melilli, Drago, & Salomone, 2013). They are classified as phytoestrogens since their structure resembles that of estrogen and they have a weak affinity for the estrogen receptor (Vaya & Tamir, 2004). These compounds have several biological activities, which confer them a beneficial potential on human health (Rietjens, Louisse, & Beekmann, 2017).

Isoflavones are usually found in nature in their glycosylated (daidzin, genistin, glycitin, puerarin) and/or methylated forms (formononetin, biochanin A). These glycosides are not absorbed at the intestine and must be hydrolysed to the aglycones daidzein, genistein and glycitein by the appropriate glycosidases and *O*-demethylases to become bioavailable and physiologically active (Gaya, Peirotén, & Landete, 2017). These transformations are carried out to a great extent by the intestinal microbiota.

The portion of aglycones that is not absorbed can be further transformed by the intestinal microbiota. In this way, daidzein can be transformed into dihydrodaidzein (DHD), *O*-desmethylangolensin (*O*-DMA) and/or equol, while genistein can originate dihydrogenistein (DHG), 6-hydroxy-*O*-DMA and/or 5-hydroxy-equol (Gaya, Peirotén, Álvarez, Medina, & Landete, 2018). These compounds produced by the intestinal microbiota are more bioavailable (Landete et al., 2016), and they have more estrogenic/antiestrogenic and antioxidant activities than their precursors (Morito et al., 2001), and have an effect in modulating hormone levels and the expression of estrogen receptors (Smeriglio, Calderaro, Denaro, Laganà, & Bellocco, 2017; Mayo, Vázquez, & Flórez, 2019).

The plasma concentration of the isoflavone compounds resulting of intestinal microbiota metabolism is subjected to large inter-individual variability (van der Velpen et al., 2014), thus hindering the establishing of the effective intake doses of soy isoflavones. In this regard, the study of the bacteria responsible for the bioactivation of isoflavones could help to understand and overcome those inter-individual differences on isoflavone metabolism (Peirotén, Bravo, & Landete, 2019).

The ability to deglycosylate isoflavones has been extensively studied from soybean extracts, soy beverages, or from pure compounds as daidzin, genistin and glicitin (Raimondi et al., 2009; Rekha & Vijayalakshmi, 2011). Lactic acid bacteria (LAB) and *Bifidobacterium* strains producing β-glucosidases have great potential for the enrichment of bioactive isoflavones daidzein and genistein in soy beverage fermentation (Pyo, Lee, & Lee, 2005; Raimondi et al., 2009). Nevertheless, large differences between strains in deglycosylation of glycosides were encountered in fermented soy beverages by LAB and *Bifidobacterium* strains (Delgado, Guadamuro, Flórez, Vázquez, & Mayo, 2019). However, the production of other metabolites derived from the metabolism of daidzein and genistein in soy beverage fermentation has not been described to date.

The demand of vegetarian alternatives for meat and dairy products is increasing, leading to the development of new soy-based products (Rizzo & Baroni, 2018). The new products based on soybeans must gather the sensory properties required by consumers, giving rise to the need of modifying soy drinks and foods containing soy extracts because of their peculiar taste (Granato, Ribeiro, Castro, & Masson, 2010). This has favored the development of soy-fermented foods containing additives to counteract soy drink strong flavours (Kaneko, Igarashi, & Aoyama, 2014). Another approach is the fermentation of soy drink, which besides of improving its sensory characteristics would increase its digestibility and the isoflavone bioavailability (Granato, Branco, Nazzaro, Cruz, & Faria, 2010).

In this work, we aimed to study the metabolism of isoflavones during the fermentation of soy drink by a selection of 20 LAB and bifidobacteria with biotechnological interest, many of which have shown the ability to metabolize different phytoestrogens or have other interesting traits (Table 1).

37 °C in RCM broth (BD, Le Pont de Claix, France). *Slackia isoflavoniconvertens* DSM22006, used as equol and 5-hydroxy-equol positive control, was grown in Wilkins-Chalgren broth (Oxoid), at 37 °C under anaerobic conditions. Identification of the strains had been previously performed by means of PCR with species-specific primers (Rodríguez et al., 2012) and confirmed by means of comparison of their 16S rRNA sequence with NCBI database. Bacterial strains were maintained at −80 °C in their respective growth media with the addition of glycerol 10% (v/v). The strains were subculture twice on their corresponding broth before their use in the experiments.

2.2. Soy drinks

Soy drinks of five different commercial brands were purchased from local supermarkets: Alpro (CAPSA, Siero, Spain), Vivesoy (Calidad Pascual, Aranda de Duero, Spain), Vital (DIA, Madrid, Spain), Hacendado (Mercadona, Tabernes Blanques, Spain) and Aliada (Hipercor, Madrid, Spain). Nutritional composition of the five different soy beverages is showed in Table 1S.

2.3. Fermentation of soy drinks by LAB and Bifidobacterium strains

LAB and *Bifidobacterium* strains were grown in their respective media during 24 and 48 h respectively under anaerobic conditions. After, 1 mL of each culture was centrifuged and the cells were resuspended in 0.5 mL of soy drink Vital. These suspensions, with concentration between $1x10^7$ and $1x10^8$ cfu/mL (determined by plating

2. Material and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study, their sources and properties are listed in Table 1. *Lactobacillus* strains were routinely cultivated at 37 °C in MRS broth (Scharlau Chemie s.a., Barcelona, Spain) under anaerobic conditions (O_2 < 0.1%; CO_2 7.0–15.0%) in sealed jars using AnaeroGen sachets (Oxoid, Ltd. Basingstoke, UK). *Lactococcus lactis* strains were grown at 30 °C in M17 broth (Scharlau Chemie, s.a.) supplemented with 0.5% glucose (Sigma-Aldrich, St Louis, MO) (GM17). *Bifidobacterium* were cultivated under anaerobic conditions at adequate decimal dilutions on the correspondent media agars and mimicking the regular concentration of commercial starter cultures used for the manufacture of fermented milks), were used to inoculate 10 mL of the same soy drink. Inoculated soy drinks were incubated during 72 h at 37 °C under anaerobic conditions. The length of the fermentation was chosen to ensure the detection of isoflavone metabolites based in preliminary assessments. All fermentations were done in duplicate. Non-inoculated soy drink was included as control.

2.4. Extraction of isoflavones

Isoflavones were extracted from the five commercial soy drinks and the fermented soy drinks, following the official AOAC method (Collison, 2008). Briefly, the reaction mixture contained 1.5 mL of the 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 PFTE membrane and the controls were filtered through a 0.45 µm PFTE membrane (Whatman; GE Healthcare Europe, Spain).

Table 1
Lactic acid bacteria and *Bifidobacterium* strains tested in this work.

Strain	Source	Trait of interest	Reference
Lactococcus			
L. lactis subsp. *lactis* INIA 415	artisanal cheese	Daidzein, DHD, genistein and DHG production.	Gaya, Peirotén, & Landete, 2017
L. lactis subsp. *lactis* ESI277	artisanal cheese	Glydosidase activity	Gaya, Peirotén, et al., 2016
L. lactis subsp. *cremoris* BO 68	artisanal cheese	Daidzein, DHD, genistein and DHG production.	Gaya, Peirotén, & Landete, 2017
Lactobacillus			
Lb. salivarius INIA P183	breast-fed infant faeces	Enterolignans production	Bravo, Peirotén, Álvarez, & Landete, 2017
Lb. rhamnosus INIA P226	breast-fed infant faeces	Biotechnological properties	Rodríguez et al., 2012
Lb. paracasei INIA P461	breast-fed infant faeces	Daidzein, DHD, genistein and DHG production	Gaya, Peirotén, & Landete, 2017
Lb. mucosae INIA P508	breast-fed infant faeces	Enterolignans production	Bravo et al., 2017
Lb. rhamnosus INIA P535	breast-fed infant faeces	SECO production from lignan extracts	Gaya, Peirotén, Medina, & Landete, 2017
Lb. rhamnosus INIA P540	breast-fed infant faeces	DHD production	Gaya, Peirotén, et al., 2016
Lb. reuteri INIA P572	pig faeces	Reuterin production	Rodríguez, Arqués, Rodríguez, Núñez, & Medina, 2003
Lb. plantarum ESI144	artisanal cheese	Biotechnological properties	Cogan et al., 1997
Bifidobacterium			
B. breve INIA P367	breast-fed infant faeces	High production of SECO from SDG.	Peirotén et al., 2019b
B. bifidum INIA P466	breast-fed infant faeces	Enterolignans production	Peirotén, Gaya, Álvarez, Bravo, & Landete, 2019
B. longum subsp. *longum* INIA P678	human milk	Daidzein, DHD, genistein and DHG production	Gaya, Peirotén, & Landete, 2017
B. catenulatum INIA P732	breast-fed infant faeces	Enterolignans production	Peirotén, Gaya, Álvarez, Bravo, & Landete, 2019
B. adolescentis INIA P784	child faeces	Daidzein, genistein, DHG and enterodiol production	Gaya, Peirotén, & Landete, 2017; Gaya, Peirotén, Medina, et al., 2017
B. pseudocatenulatum INIA P815	adult faeces	Urolthins A and B production.	Gaya, Peirotén, Medina, Álvarez, & Landete, 2018b
B. dentium INIA P886	adult faeces	Glycosidase activity	INIA culture collection
B. animalis INIA P900	adult faeces	Daidzein, DHD, genistein and DHG production.	Gaya, Peirotén, & Landete, 2017
B. pseudocatenulatum INIA P946	breast-fed infant faeces	High production of SECO from SDG.	Peirotén, Gaya, Álvarez, Bravo, & Landete, 2019

DHD, dihydrodaidzein ;DHG, dihydrogenistein; SECO, secoisolariciresinol; SDG, secoisolariciresinol diglucoside

2.5. Quantification of main isoflavones in commercial soy drinks

The glucosides daidzin and genistin and the aglycones daidzein and genistein were quantified in the five commercial soy drinks by HPLC-PAD according to Gaya, Arqués, Álvarez, Medina, and Landete (2016).

2.6. Identification and quantification of isoflavones produced by LAB and Bifidobacterium strains in fermented soy drink.

The analyses were performed by means of HPLC–ESI/MS and HPLC-ESI/MS/MS (Gaya, Peirotén, Álvarez, et al., 2018). Briefly, analyses were carried out using an Agilent 1200 series LC, comprised of quaternary pump with integrated degasser, thermostated autosampler,

Table 2
Isoflavones concentration (µM) in commercial soy drinks.

	Daidzin	Daidzein	Genistin	Genistein
Vital	317.1 ± 9.7^{c}	4.8 ± 1.0^{a}	462.1 ± 12.8^{c}	3.7 ± 0.5^{a}
ViveSoy	101.9 ± 4.3^{a}	3.8 ± 0.6^{a}	282.8 ± 9.1^{ab}	4.2 ± 0.7^{a}
Hacendado	119.0 ± 2.8^{ab}	2.2 ± 0.4^{a}	235.7 ± 13.2^{a}	3.0 ± 1.0^{a}
Alpro	110.6 ± 5.6^{ab}	2.9 ± 0.2^{a}	310.0 ± 5.8^{ab}	3.9 ± 0.5^{a}
Aliada	136.1 ± 1.9^{b}	2.6 ± 1.0^{a}	321.2 ± 21.2^{b}	3.0 ± 0.1^{a}

$^{a-c}$ Values in the same column with different superscript differ significantly (P < 0.01).

the highest concentration of genistin and daidzin compared with the rest of beverages (P > 0.01). All of them presented also low amounts of daidzein and genistein, with no significant differences among them. Based on these results, the soy drink Vital was chosen for the fermentation assays by LAB and *Bifidobacterium* strains. All the strains tested caused a decreased in the pH after fermentation, ranging from 3.7 to 5.1 (data not shown), compared with the control (pH 6.9–7.1) and showing curdling of the soymilk.

thermostated column compartment and diode array detector, coupled with an Agilent 6530 Accurate-Mass Quadrupole Time of Flight (Q-TOF) LC/MS with ESI-Jet Stream Technology (Agilent Technologies). The Q-TOF acquisition method was 4 GHz, mass range low 1700 *m/z*, negative polarity, drying gas 10 L/min and 350 °C, sheath gas 11 L/min and 350 °C, nebulizer 45 psi, capillary voltage 3500 V, nozzle voltage 0 V and fragmentor voltage 120 V. For targeted MS/MS analysis, 20 V collision energy was used. Data acquisition (version B.05.01) and Qualitative Analysis (version B.07.00) of MassHunter Workstation Software were used (Agilent Technologies, Waldbroon, Germany).

Identification of isoflavone compounds was based on comparison of their retention times and mass spectral data with those of standards. Daidzein, daidzin, equol, genistein, genistin, resorcinol, phloroglucinol and 2-(4-hydroxyphenyl)-propionic acid were purchased from LC Laboratories (Woburn, MA). DHD and DHG were purchased from Toronto Research Chemicals (Toronto, Canada). Stock solutions of those standards were prepared in DMSO (Sigma-Aldrich) in a concentration of 10 mg/L. *O*-DMA and 6-hydroxy *O*-DMA were tentatively characterized by means of the interpretation of the observed MS/MS spectra according to Gaya, Peirotén, Álvarez, et al. (2018). Whereas, tetrahydrodaidzein (THD) was tentatively characterized by means of the interpretation of the observed MS/MS spectra according the THD production of *Slackia isoflavoniconvertens* DSM22006. Quantification was made by means of external standard calibration curves. *O*-DMA, THD and 6-hydroxy *O*-DMA were quantified using the calibration curves of the most similar compounds DHD and DHG.

2.7. Statistical analysis

Statistical analysis of the isoflavone concentrations was performed using of SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, USA). Data were analysed by ANOVA using a general linear model (GLM). Comparison of means was carried out by Tukey test, with a confidence interval of 99%.

3. Results

3.1. Concentration of isoflavones in soy drinks

In order to select one soy drink for the fermentation experiments, we analyzed the concentration of isoflavones in five different commercial soy drinks. The main isoflavone glucosides, daidzin and genistin, and their respective aglycones, daidzein and genistein were quantified, observing important differences between the isoflavone concentrations of each soy drink (Table 2). The soy drink Vital showed

3.2. Daidzin metabolism by LAB and Bifidobacterium strains in fermented soy drink

The majority of the strains tested were able to convert daidzin to its aglycone daidzein (Table 3). All the *Bifidobacterium* strains showed higher daidzein and lower daidzin than the control, in particular *B. breve* INIA P367, which showed the highest daidzein and lowest daidzin levels, suggesting a high efficiency in its deglycosylation. *Lactobacillus* strains showed also deglycosidation activity, although a couple of them did not differ statistically from the controls. Conversely, *Lactococcus* strains showed low efficiency in the deglycosylation of daidzin.

HPLC-ESI/MS analyses revealed the DHD production in low concentration in LAB and *Bifidobacterium* strains. Moreover, a peak with retention time 24.3 and molecular [M−H]- ions at *m/z* 255.0663 was also detected (Table 3), corresponding with a molecular formula ($C_{15}H_{12}O_4$) similar to that of DHD (retention time of DHD 23.5). The strains that produced DHD also produced this compound in similar concentrations, with the exception of *B. longum* INIA P678, which only showed the presence of the peak with retention time 24.3.

ESI/MS analyses revealed two peaks with retention times 34.8 and 35.3 and molecular [M−H]- ions at *m/z* 257.0819 corresponding to both *O*-DMA and THD. Since authentic reference compounds were not available, identification was accomplished by interpretation of the MS/MS data. The identification of *O*-DMA was also confirmed by comparison with the spectra of the *O*-DMA produced by *Enterococcus faecium* INIA P553 and previously described by Gaya, Peirotén, Álvarez, et al. (2018). The peak assigned to TDH showed the same spectra as the TDH produced by *S. isoflavoniconvertens* DSM22006, a strain able to transform daidzein into equol via DHD and TDH (Schröder, Matthies, Engst, Blaut, & Braune, 2013) (Fig. 1).

THD was detected in half of the fermented soy drinks. *B. pseudocatenulatum* INIA P815 showed the highest THD production. None of the strains tested was able to produce equol. Many of the *Bifidobacterium* and *Lactobacillus* strains, together with *L. lactis* 415, were capable of producing *O*-DMA. In this case, the highest *O*-DMA levels were achieved by two lactobacilli, *Lb. mucosae* INIA P508 and *Lb. plantarum* ESI144

3.3. Genistin metabolism by LAB and Bifidobacterium strains

LAB and *Bifidobacterium* strains showed a greater deglycosylation of genistin than that of daidzin, with greater decreases in the genistin levels compared to control (Table 4). Among the strains tested, *Bifidobacterium* strains showed greater efficiency in the transformation of genistin, especially *B. breve* INIA 367, *B. bifidum* INIA P466, and *B.*

Table 3
Daidzin transformation in fermented soy drink by LAB and Bifidobacterium strains. Compound concentrations are expressed in μM.

Strains	Daidzin	Daidzein	DHD-DHD*	THD	*O*-DMA
Control t = 0	317.53 ± 2.67 ^{h}	4.85 ± 0.87 ^{a}	n.d	n.d.	n.d.
Control T = 72 h	309.21 ± 8.13 ^{gh}	8.91 ± 4.65 ^{ab}	n.d	n.d.	n.d.
Lactococcus					
L. lactis subsp.*lactis* 415	238.39 ± 56.76 ^{efgh}	46.56 ± 14.76 ^{abc}	0.83 ± 0.21 ^{a}	n.d.	4.32 ± 1.25 ^{a}
L. lactis subsp.*lactis* ESI277	289.02 ± 23.09 ^{fgh}	9.27 ± 6.32 ^{ab}	n.d.	n.d.	n.d.
L. lactis subsp.*cremoris* Bo68	125.15 ± 61.89 ^{abcde}	93.57 ± 34.21 ^{abcde}	1.71 ± 0.82 ^{ab}	n.d.	n.d.
Lactobacillus					
Lb. salivarius INA P183	154.93 ± 85.98 ^{bcdef}	129.28 ± 17.86 ^{cde}	n.d.	n.d.	n.d.
Lb. rhamnosus INIA P226	72.80 ± 34. 21 ^{abcd}	119.93 ± 31.21 ^{bcde}	2.35 ± 1.01 ^{ab}	7.8 ± 4.81 ^{ab}	2.01 ± 1.45 ^{a}
Lb. paracasei INIA P461	115.06 ± 19.21 ^{abcde}	50.30 ± 11.72 ^{abc}	1.33 ± 0.89 ^{ab}	11.2 ± 1.67 ^{ab}	n.d.
Lb. mucosae INIA P508	37.06 ± 11.02 ^{abcd}	178.39 ± 9.92 ^{de}	3.08 ± 0.67 ^{ab}	14.89 ± 2.56 ^{ab}	21.32 ± 1.21^{c}
Lb. rhamnosus INIA P535	15.34 ± 8.01 ^{ab}	201.72 ± 6.21 ^{ef}	n.d.	n.d.	4.76 ± 0.89 ^{a}
Lb. rhamnosus INIA P540	83.46 ± 34.01 ^{abcd}	167.04 ± 31.82 ^{de}	1.50 ± 0.52 ^{ab}	1.13 ± 0.09 ^{a}	2.69 ± 0.98 ^{a}
Lb. reuteri INIA P572	177.54 ± 22.08 ^{defgh}	74.75 ± 24.32 ^{abcd}	n.d.	n.d.	n.d.
Lb. plantarum ESI144	172.82 ± 11.07 ^{cdefg}	71.77 ± 17.07 ^{abcd}	0.67 ± 0.42 ^{a}	3.98 ± 1.11 ^{a}	21.08 ± 3.21^{c}
Bifidobacterium					
B. breve INIA P367	1.11 ± 0.81 ^{a}	291.35 ± 21.22^{f}	n.d.	n.d.	n.d.
B. bifidum INIA P466	79.85 ± 7.05 ^{abcd}	166.27 ± 42.12 ^{de}	n.d.	n.d.	4.78 ± 0.12 ^{a}
B. longum INIA P678	46.79 ± 11.17 ^{abcd}	200.16 ± 12.05 ^{ef}	1.95 ± 0.32 ^{ab}	1.54 ± 0.32 ^{a}	8.91 ± 3.81 ^{a}
B. catenulatum INIA P732	31.61 ± 13.92 ^{abc}	175.52 ± 45.23 ^{de}	n.d.	n.d.	17.91 ± 1.11 ^{bc}
B. adolescentis INIA P784	56.82 ± 19.81 ^{abcd}	171.51 ± 11.09 ^{de}	0.74 ± 0.41 ^{a}	2.76 ± 1.01 ^{a}	1.78 ± 0.56 ^{a}
B. pseudocatenulatum INIA P815	22.83 ± 9.13 ^{ab}	191.58 ± 33.08 ^{ef}	4.56 ± 1.35^{b}	21.72 ± 7.09^{b}	7.89 ± 2.09 ^{a}
B. dentium INIA P886	81.61 ± 13.81 ^{abcd}	166.94 ± 16.28 ^{de}	1.21 ± 0.07 ^{ab}	1.32 ± 0.43 ^{a}	10.34 ± 1.34 ^{ab}
B. animalis INIA P900	39.11 ± 4.51 ^{abcd}	179.63 ± 9.01 ^{de}	0.77 ± 0.48 ^{a}	9.98 ± 3.17 ^{ab}	n.d.
B. pseudocatenulatum INIA P946	7.73 ± 0.92 ^{a}	186.55 ± 28.91 ^{ef}	1.82 ± 1.14 ^{ab}	1.78 ± 0.46 ^{a}	2.82 ± 0.33 ^{a}

DHD*, dihydrodaidzein isomer; The concentrations shown refer to the DHD*, similar concentrations of DHD are suggested, with the exception of *B. longum* INIA P678 which only showed the presence of DHD*. TDH, tetrahydrodaidzein; *O*-DMA, *O*-desmethylangolensin; n.d. not detected; $^{a-h}$ values in the same column with different superscript differ significantly ($P < 0.01$).

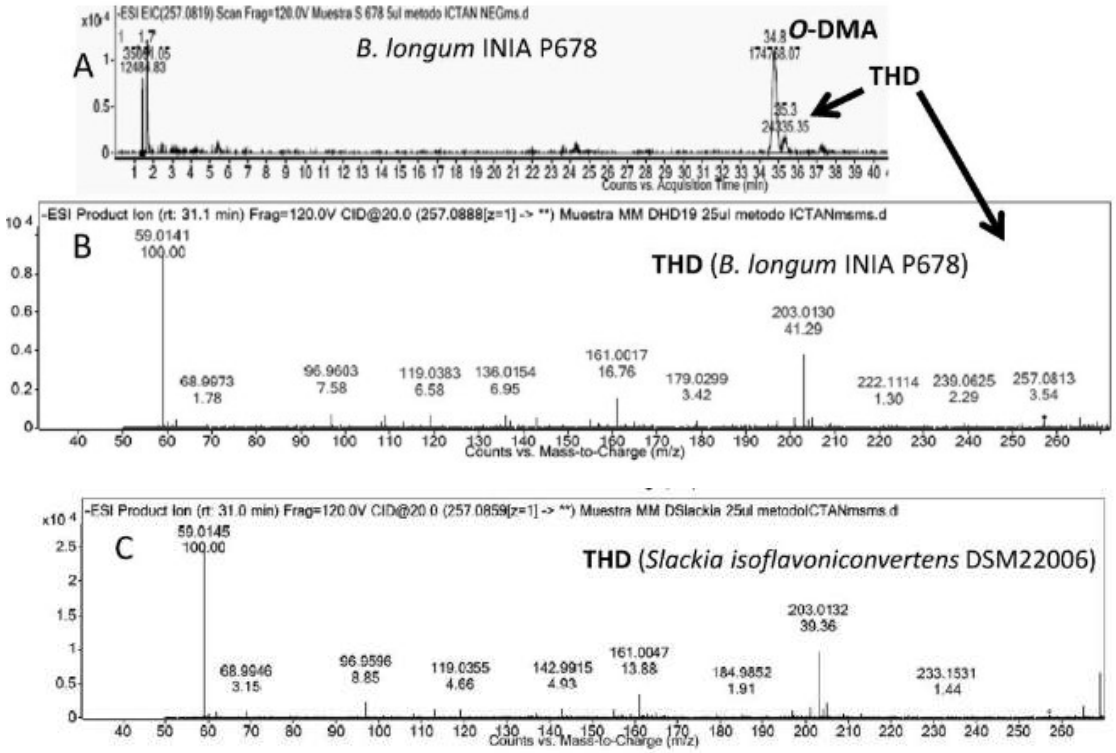

Fig. 1. Production of *O*-desmethylangolensin (*O*-DMA) and tetrahydrodaidzein (THD) in fermented soy milk. (A) *O*-DMA and THD detected by HPLC-ESI/MS after soy beverage fermentation with *B. longum* INIA P678. (B) HPLC-ESI/MS/MS spectra of THD produced by *B. longum* INIA P678. (C) HPLC-ESI/MS/MS spectra of THD produced by *S. isoflavoniconvertens* DSM22006 from dihydrodaidzein (Gaya, Peirotén, Álvarez, et al., 2018). (B).

Table 4
Genistin and glycitin metabolism by LAB and *Bifidobacterium* strains in fermented soy drink. Compounds concentrations are expressed in μM.

Strains	Genistin	Genistein	6-OH-*O*-DMA	2-(4-hydroxyphenyl) propionic acid	Glycitin	Glycitein
Control t = 0	462.11 ± 11.20 ^{g}	3.74 ± 0.21 ^{a}	n.d.	10.54 ± 3.45 ^{a}	2.49 ± 0.52 ^{a}	n.d.
Control T = 72 h	458.19 ± 9.17 ^{g}	4.54 ± 1.28 ^{a}	n.d.	11.71 ± 2.12 ^{a}	2.51 ± 0.63 ^{a}	0.17 ± 0.09 ^{a}
Lactococcus						
L. lactis subsp.*lactis* 415	382.77 ± 23.91 ^{fg}	27.94 ± 9.82 ^{a}	21.62 ± 9.91 ^{a}	28.65 ± 12.78 ^{a}	1.74 ± 0.45 ^{a}	0.09 ± 0.07 ^{a}
L. lactis subsp.*lactis* ESI277	427.11 ± 12.09 ^{g}	6.52 ± 4.81 ^{a}	n.d.	17.02 ± 8.09 ^{a}	1.92 ± 1.01 ^{a}	n.d.
L. lactis subsp.*cremoris* NBo68	314.37 ± 34.07^{f}	81.49 ± 18.92 ^{abc}	13.72 ± 7.83 ^{a}	21.91 ± 3.45 ^{a}	1.41 ± 0.47 ^{a}	0.42 ± 0.21 ^{ab}
Lactobacillus						
Lb. salivarius INA P183	140.72 ± 21.91 ^{bcd}	142.84 ± 65.91 ^{abcd}	1.23 ± 1.01 ^{a}	12.87 ± 2.67 ^{a}	n.d.	1.58 ± 0.82 ^{ab}
Lb. rhamnosus INIA P226	118.32 ± 31.18 ^{abc}	117.88 ± 42.11^{abc}	4.81 ± 0.91 ^{a}	52.43 ± 11.52 ^{ab}	n.d.	1.35 ± 0.31 ^{ab}
Lb. paracasei INIA P461	301.98 ± 23.83 ^{ef}	57.34 ± 16.54^{ab}	11.92 ± 3.65 ^{a}	115.42 ± 36.67 ^{ab}	1.29 ± 0.21 ^{a}	0.33 ± 0.10 ^{ab}
Lb. mucosae INIA P508	68.65 ± 15.90 ^{abc}	280.40 ± 19.92^{d}	0.72 ± 0.11 ^{a}	45.14 ± 3.56 ^{ab}	n.d.	1.32 ± 0.54 ^{ab}
Lb. rhamnosus INIA P535	116.54 ± 17.16 ^{abc}	189.34 ± 12.82^{bcd}	1.52 ± 0.91 ^{a}	201.91 ± 56.61 ^{ab}	n.d.	1.58 ± 0.11 ^{ab}
Lb. rhamnosus INIA P540	114.25 ± 19.21 ^{abc}	220.92 ± 9.96 ^{cd}	8.88 ± 1.03 ^{a}	128.01 ± 5.91 ^{ab}	n.d.	1.28 ± 0.42 ^{ab}
Lb. reuteri INIA P572	216.73 ± 34.89 ^{de}	67.72 ± 18.91^{ab}	24.22 ± 7.65 ^{a}	78.93 ± 17.39 ^{ab}	n.d.	1.03 ± 0.65 ^{ab}
Lb. plantarum ESI144	162.97 ± 28.92 ^{cd}	86.45 ± 47.81^{abc}	1.93 ± 0.67 ^{a}	165.11 ± 23.53 ^{ab}	1.11 ± 0.64 ^{a}	0.58 ± 0.09 ^{ab}
Bifidobacterium						
B. breve INIA P367	n.d.	284.52 ± 68.91^{d}	31.76 ± 12.72 ^{a}	60.94 ± 17.8 ^{ab}	n.d.	0.09 ± 0.08 ^{a}
B. bifidum INIA P466	n.d.	272.59 ± 45.34 ^{d}	21.89 ± 7.98 ^{a}	176.55 ± 101.72 ^{ab}	n.d.	0.30 ± 0.21 ^{ab}
B. longum INIA P678	52.31 ± 14.91 ^{ab}	188.83 ± 18.21 ^{bcd}	15.21 ± 9.31 ^{a}	132.10 ± 25.32 ^{ab}	n.d.	1.45 ± 1.01 ^{ab}
B. catenulatum INIA P732	28.49 ± 9.08 ^{a}	172.80 ± 34.81 ^{bcd}	9.11 ± 4.89 ^{a}	182.41 ± 65.43 ^{ab}	n.d.	0.71 ± 0.23 ^{ab}
B. adolescentis INIA P784	49.42 ± 11.21 ^{ab}	173.04 ± 23.93 ^{bcd}	10.82 ± 6.01 ^{a}	86.13 ± 17.72 ^{ab}	n.d.	0.08 ± 0.05 ^{a}
B. pseudocatenulatum INIA P815	52.34 ± 7.12 ^{ab}	222.43 ± 11.34 ^{cd}	21.23 ± 10.05 ^{a}	246.27 ± 67.98^{b}	n.d.	2.17 ± 0.16^{b}
B. dentium INIA P886	34.19 ± 2.46 ^{a}	178.84 ± 9.91 ^{bcd}	25.12 ± 16.04 ^{a}	205.04 ± 25.67 ^{ab}	n.d.	0.67 ± 0.31 ^{ab}
B. animalis INIA P900	30.87 ± 6.92 ^{a}	278.52 ± 21.01 ^{d}	0.11 ± 0.08 ^{a}	161.43 ± 101.18 ^{ab}	n.d.	0.49 ± 0.16 ^{ab}
B. pseudocatenulatum INIA P946	n.d.	276.37 ± 8.12 ^{d}	14.21 ± 1.03 ^{a}	216.53 ± 59.91 ^{ab}	n.d.	0.73 ± 0.34 ^{ab}

6OH-*O*-DMA, 6-hydroxy-*O*-desmethylangolensin; n.d. not detected; $^{a-g}$ values in the same column with different superscript differ significantly ($P < 0.01$).

pseudocatenulatum INIA P946, which metabolized all the genistin. The *Lactococcus* strains showed the lowest efficiency in the deglycosylation of genistin in soy drinks.

We did not detect DHG in any of the strains studied, however, most of the strains showed production of 6-hydroxy-*O*-DMA. Analysis of the peak that exhibited a [M−H]- ion at *m/z* 273.0768 and retention time 20,3 coincided with that of the compound identified as 6-hydroxy-*O*-DMA in the metabolism of genistein by *E. faecium* INIA P553 (Gaya, Peirotén, Álvarez, et al., 2018). Additionally, we found increased concentrations of 2-(4-hydroxyphenyl)-propionic acid compared to the control in the majority of the fermented soy drinks analyzed (Table 4), although only *B. pseudocatenulatum* INIA P815 showed a value statistically higher that the control. This compound has been described as final product of the degradation of genistein (Schoefer, Mohan, Braune, Birringer, & Blaut, 2002).

3.4. Glycitin metabolism by LAB and Bifidobacterium strains

The concentration of glycitin in soy drink was much lower than that of daidzin and genistin. After fermentation, the majority of *Lactobacillus* and all *Bifidobacterium* strains metabolized all glycitin (Table 4) producing glycitein, although the levels observed were statistically higher only for *B. pseudocatenulatum* INIA P815. We did not observe the formation of peaks corresponding to dehydroglycitein and 5-methoxy-*O*-desmethylangolensin.

4. Discussion

Isoflavones found in plant and soy drinks are usually glycosylated and/or methylated, and are hydrolysed to the aglycones daidzein, genistein and glycitein by the appropriate glycosidases and *O*-demethylases to become bioavailable (Larkin, Price, & Astheimer, 2008). As expected, the isoflavones measured in the different commercial soy drinks were mainly in the form of glycosides (Table 2). The aglycones daidzein and genistein represented a small percentage of the total concentration of isoflavones in the commercial soy drinks, in concordance with Delgado et al. (2019). As shown in Table 2, there are evident differences in the concentration of isoflavones in the different commercial soy drinks analyzed, which could lead to different degree of effects on consumer health.

Published works have explored the fermentation of soy drinks by lactobacilli and bifidobacteria strains in order to improve the bioavailability of isoflavones in the product, alongside with the increase of B vitamins or the reduction of the levels of antinutrients (Raimondi et al., 2009; Rekha & Vijayalakshmi, 2011). Besides daidzein, genistein and glycitein production, we aimed to analyze also the production of other compounds resulting of the bacterial metabolism of isoflavones in fermented soy drinks, such as DHD, *O*-DMA, equol, DHG and 6-hydroxy-*O*-DMA. LAB and *Bifidobacterium* strains, which had previously shown ability to metabolize isoflavones, ellagitannins and/or lignans (Table 1), were selected to assess their metabolic activity on isoflavones during soy drink fermentation.

Genistin seemed to be more susceptible to deglycosylation by bifidobacteria and lactobacilli than daidzin, although the higher initial levels could facilitate its metabolization. The strains *B. breve* INIA P367, *B. pseudocatenulatum* INIA P946 and *B. bifidum* INIA P466 showed the complete deglycosylation of genistin. Interestingly, the two first bifidobacteria had showed also high glycosylase activity in the transformation of secoisolariciresinol diglucoside into secoisolariciresinol (Peirotén, Gaya, Álvarez, Bravo, & Landete, 2019). On the contrary, *Lactococcus* strains, that had showed greater activity in the deglycosylation of pure compounds of daidzin and genistin in culture medium (Gaya, Peirotén, & Landete, 2017), showed low levels of deglycosidation in soy drink. Thus, the medium and the growth conditions of the bacteria might influence their metabolism of isoflavones.

After deglycosylation, the proposed routes for the metabolism of isoflavone aglycones result in the formation of *O*-DMA or equol from daidzein via DHD, and 6-hydroxy-*O*-DMA or 5'hydroxyl-equol from genistein via DHG (Heinonen, Hoikkala, Wähälä, & Adlercreutz, 2003). Several of the LAB and *Bifidobacterium* strains tested had shown DHD production in culture medium (Gaya, Peirotén, et al., 2016; Gaya, Peirotén, & Landete, 2017). However, after soy beverage fermentation, those same strains produced DHD in lower concentration and a peak with a similar molecular mass to DHD but with different retention time (24.3), although this retention time appeared close to the retention time of DHD (retention time 23.5). These data agreed with the observed production of THD, since THD is produced from DHD by the action of enzymes with similar action to dihydrodaidzein reductase. The strains that produced DHD showed a similar production of the unidentified peak at retention time 24.3, with the exception of *B. longum* INIA P678. That is in agreement with the results of Shimada et al. (2012), regarding the activity of the dihydrodaidzein racemase from *Lactococcus* strain 20–92. According to that, we suggest that the unidentified peak at 24.3 toghether with the DHD peak could correspond to each one of the (R)- and (S)-enantiomers of DHD.

While most of the tested strains were capable of producing DHD, DHG could not be detected in any fermented soy drink. This finding could indicate that the conversion of genistein to 6- hydroxy *O*-DMA by these strains during fermentation of soy drink could occur through intermediates different to DHG. The absence of DHG as intermediate of genistein metabolization has been described in *Eubacterium ramulus* (Schoefer et al., 2002). However, we had observed previously the production of DHG from genistin in culture media (Gaya, Peirotén, & Landete, 2017) by several of the strains tested in this work (Table 1). The lack of DHG in fermented soy drink but not in culture media could indicate an influence of external conditions in the metabolism of genistin by those bacteria.

Many of the *Lactobacillus* and *Bifidobacterium* strains were able to produce THD in low amounts (Fig. 1). Although THD is a precursor of equol, we did not detect equol production in any of the fermented soy drinks. The production of equol in fermented soy milk has been described previously and could depend on the composition of the soy milk (Di Cagno et al., 2010). The *Lactobacillus* and *Bifidobacterium* strains tested that produce TDH may lack an enzyme similar to the tetrahydrodaidzein reductase from *S. isoflavoniconvertens*, which allows the transformation of TDH into equol (Schröder et al., 2013). Subsequent studies would be carried out to explore the possibilities of increasing the levels of TDH, as a way to facilitate later formation of equol.

Many of the LAB and *Bifidobacterium* strains were able to produce *O*-DMA and/or 6-hydroxy-O-DMA in soy drink. *O*-DMA has shown several biological activities in vitro, including estrogenic/antiestrogenic and antiproliferative effects (Frankenfeld, 2011), and has been associated with favorable cardiovascular risk profiles (Liu, Ho, Chen, Liu, & Woo, 2014). The production of *O*-DMA and 6-hydroxy-*O*-DMA from daidzin hydroxyphenyl) propionic acid surpassed the amounts of deglycosilated genistin, that is the difference between initial genistin and the sum of the remaining genistin and subsequent metabolites was below the obtained 2-(4-hydroxyphenyl) propionic acid. This could suggest the existence of other precursors for this compound. In the case of daidzin and its subsequent metabolites, we also observed an imbalance between the disappeared daidzin and the detected daidzein, DHD isomer, THD and *O*-DMA. It could suggest that 2-(4-hydroxyphenyl) propionic acid could also be a result of *O*-DMA degradation. In that case, resorcinol would have to be produce alongside 2-(4-hydroxyphenyl) propionic acid, as a result of the cleavage of *O*-DMA (Fig. 1S). Likewise to phloroglucinol, resorcinol standard was not detectable by HPLC unless high concentrations were used (data not shown). In the case of *E. ramulus*, *O*-DMA was not further degraded, indicating that the 6-hydroxyl group of 6́-hydroxy-*O*-desmethylangolensin is a prerequisite for cleavage by this organism (Schoefer et al., 2002), although it could be different for some of the LAB and bifidobacteria tested in this work. Another possibility would be the production of 2-(4-hydroxyphenyl) propionic acid from other compounds, such as other flavonoids, present in soy, although their concentration is low compared with the concentration of isoflavones.

In this work we present some LAB and *Bifidobacterium* strains able to metabolize the glycoside isoflavones present in commercial soy drink, producing several isoflavone derivates, including the bioactive aglycones daidzein and genistein, *O*-DMA, 6-hydroxy-*O*-DMA and THD (Fig. 1S). Some of these strains have previously shown the ability to bioactivate other phytoestrogens (Table 1) and could be used for the development of fermented products enriched with enterolignans and urolithins A and B. Moreover, many of the lactobacilli and bifidobacteria species are good candidates to be added to food products, since they are considered by the European Food Safety Authority (EFSA) to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment (EFSA, 2017). Further physicochemical and sensory characterization of the fermented products, together with the present results on isoflavones bioactivation, would allow the selection of the more interesting LAB and bifidobacterial strains.

and genistin respectively, was described in an *Enterococcus faecium* strain (Gaya, Peirotén, Álvarez, et al. (2018)), while several strains placed in the Clostridiales order are also able of producing *O*-DMA (Li et al., 2015; Schoefer et al., 2002; Yokoyama, Niwa, Osawa, & Suzuki, 2010). This is the first time that the production of *O*-DMA, an isoflavone with potential effect on human health (Liu et al., 2014; Frankenfeld, 2011), is detected in soy drink fermented by LAB or bifidobacteria.

In the same way that daidzein can be transformed into DHD and *O*-DMA and genistein can be transformed into DHG and 6-hydoxy-*O*-DMA, glycitein could be transformed into or dehydroglycitein and 5-methoxy-*O*-desmethylangolensin respectively. However, we did not detect the formation of these compounds. LAB and *Bifidobacterium* strains may not be able to produce them. Nevertheless, it is also possible that the low concentration of glycitin present in soy drink did not allow us to detect other compounds derived from glycitin.

Additionally to the described isoflavone derivates, we also detected 2-(4-hydroxyphenyl) propionic acid in the soy drinks fermented by LAB and bifidobacteria (Table 4). This compound has been described as a degradation product of genistein metabolism by *E. ramulus*, which transform 6-hydroxy-*O*-DMA into phloroglucinol and 2-(4-hydroxyphenyl) propionic acid (Schoefer et al., 2002). Nevertheless, phloroglucinol was not detected in our samples. One possible reason could be a quick degradation of this compound according to Schoefer et al. (2002). We also observed that the phloroglucinol standard was only detectable at high concentrations, suggesting the lack of suitability of our method for the quantification of this compound. Hence, low levels of this compound in our samples would not have been detected.

In some of the fermented soy drinks, the amounts of 2-(4-

5. Conclusions

LAB and *Bifidobacterium* strains metabolized the isoflavones present in commercial soy drink, producing daidzein, genistein, DHD, THD, *O*-DMA and 6 hydroxy *O*-DMA. This is the first time that THD, *O*-DMA and 6-hydroxy *O*-DMA are described as a result of soy drink fermentation. These strains have a promising biotechnological potential since they could allow an economical and environmentally friendly way to enrich soy drink with bioactive isoflavones, such as the aglycones and *O*-DMA, or the precursor of equol, THD. Moreover, the previously reported ability of some of the lactobacilli and bifidobacteria tested to bioactivate other phytoestrogens, open the possibility to explore the development of soy drinks enriched with enterolignans or urolithins.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by project RTA2017-00002-00-00 from the Spanish Ministry of Science, Innovation and Universities. We are grateful to the ICTAN (Institute of Food Science, Technology and Nutrition, Madrid, Spain) Analysis Services Unit for providing chromatography and mass spectrometry facilities.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126521.

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