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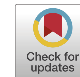

Heterologous production of equol by lactic acid bacteria strains in culture medium and food

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ABSTRACT

The isoflavones daidzin and genistin, present in soybeans, can be transformed by the intestinal microbiota into equol and 5-hydroxy-equol, compounds with enhanced availability and bioactivity, although these are only produced by a fraction of the population. Hence, there is an interest in the production of these compounds, although, to date, few bacteria with biotechnological interest and applicability in food have been found able to produce equol. In order to obtain lactic acid bacteria able to produce equol, the daidzein reductase (*dzr*), dihydrodaidzein reductase (*ddr*), tetrahydrodaidzein reductase (*tdr*) and dihydrodaidzein racemase (*ifc*A) genes, from *Slackia isoflavoniconvertens* DSM22006, were cloned into the vector pNZ:TuR, under a strong constitutive promoter (TuR). *Lactococcus lactis* MG1363, *Lacticaseibacillus casei* BL23, *Lactiplantibacillus plantarum* WCFS1, *Limosilactobacillus fermentum* INIA 584L and *L. fermentum* INIA 832L, harbouring pNZ:TuR.tdr.ddr, were able to produce equol from dihydrodaidzein, while *L. fermentum* strains showed also production of 5-hydroxy-equol from dihydrogenistein. The metabolization of daidzein and genistein by the combination of strains harbouring pNZ:TuR.dzr and pNZ:TuR.tdr.ddr showed similar results, and the addition of the correspondent strain harbouring pNZ:TuR.ifcA resulted in an increase of equol production, but only in the *L. fermentum* strains. This pattern of equol and 5-hydroxy-equol production by *L. fermentum* strains was also confirmed in cow's milk supplemented with daidzein and genistein and incubated with the different combination of strains harbouring the constructed plasmids. Bacteria generally recognized as safe (GRAS), such as the lactic acid bacteria species used in this work, harbouring these plasmids, would be of value for the development of fermented vegetal foods enriched in equol and 5-hydroxy-equol.

1. Introduction

Isoflavones are flavonoids present in various plants, particularly in soybean germ (Aguiar et al., 2007). They are classified as phytoestrogens since their structures resemble that of estrogen and have weak affinity for the estrogen receptor (Vaya and Tamir, 2004). Due to their biological activity, isoflavones have been linked to beneficial effects in health, including positive effects in ameliorating menopause symptoms and reducing the risk of cardiovascular disease and certain types of cancer (Ko, 2014; Mayo et al., 2019). Isoflavones are usually found in nature in their glycosylated form, which are not absorbed on the intestine, being daidzin and genistin the most abundant in soy. Glycosides must be hydrolyzed to aglycones (daidzein and genistein), by the appropriate glycosidases, to become bioavailable and physiologically active (Gaya et al., 2017; Setchell et al., 2002). Then, daidzein and genistein can be converted into dihydrodaidzein (DHD) and dihydrogenistein (DHG) by means of hydrogenation reaction. After this, the compounds *O*-desmethylangolensin (*O*-DMA) and/or equol can be formed from DHD by means of ring-cleavage or keto-elimination reactions, respectively. In the same way, DHG can be transformed into 6-hydroxy-*O*-DMA or 5-hydroxy-equol (Gaya et al., 2018).

Equol is the isoflavone derivate with the highest estrogenic and antioxidant activity, it is characterized by being more stable than its isoflavone precursor (Lephart, 2019) and thus, there is an increasing interest in unraveling its influence in health (Mayo et al., 2019). Nevertheless, only a 30–50% of the western adult population produce equol, while production of *O*-DMA is much more extended (Atkinson et al., 2005). On its part, 5-hydroxy-equol, has been less studied, although it has more antioxidant activity than its precursor genistein (Arora et al., 1998) and has shown anticarcinogenic activity in vitro

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(Gao et al., 2018).

The interesting properties of equol have led to the searching of equol-producing bacteria of intestinal origin. In addition to bacterial combinations, strains capable of completing the transformation of daidzein into equol have been identified, mainly belonging to the family *Eggerthellaceae*, such as *Adlercreutzia equolifaciens*, *Assacharobacter celatus*, *Eggerthella* spp., *Enterorhabdus mucosicola*, *Slackia isoflavoniconvertens* and *Slackia equolifaciens* (Mayo et al., 2019). Besides these species, some intestinal lactic acid bacteria (LAB) strains have been described as equol-producers, i.e. *Lactobacillus intestinalis* (Heng et al., 2019) and *Lactococcus garvieae* 20–92 (Uchiyama et al., 2007). However, to date no bacteria with biotechnological interest, such as the LAB species commonly used as food fermentation starters or as probiotics, have been found able to produce equol from daidzein.

The enzymes involved in equol synthesis from daidzein are the daidzein reductase (DZNR), the DHD reductase (DHDR) and the tetrahydrodaidzein reductase (THDR) (Wang et al., 2005). A DHD racemase (DDRC) has been also described to play an important role in the production of equol, since the preferred substrate of DHDR is S-DHD but the main product of DZNR seems to be R-DHD and, thus, the racemase activity transforming the enantiomers favors the equol production (Shimada et al., 2012). The genes encoding those enzymes have been described to be organized in a cluster with similar sequence and genetic organization between different equol-producing strains such as *S. isoflavoniconvertens* DSM 22006, *Eggerthella* sp. YY7918 and L. *garvieae* 20-92 (Kawada et al., 2016).

The objective of this work was to produce equol and 5-hydroxy-equol by bacteria suitable for their use in fermented food, such as species of LAB generally recognized as safe (GRAS) and with qualified presumption of safety (QPS) status. The DZNR gene (*dzr*) had been previously cloned into different LAB and *Bifidobacterium* strains observing DHD and DHG production in fermented soy beverage (Peirotén et al., 2020b). In this work, the DHDR gene (*ddr*), THDR gene (*tdr*) and DDRC gene (*ifc*A) were cloned and transformed into different LAB strains, including the species *Lactococcus lactis, Limosilactobacillus fermentum* (previously *Lactobacillus fermentum), Lacticaseibacillus casei* (previously *Lactobacillus casei*) and *Lactiplantibacillus plantarum* (previously *Lactobacillus plantarum*). The production of equol and 5-hydroxy-equol from different precursors in culture medium and in a food matrix was studied.

2. Material and methods

2.1. Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. *L. lactis* MG1363 was grown at 30 °C in M17 broth (Scharlau Chemie, SA, Barcelona, Spain) supplemented with 0.5% glucose (Merck KGaA, Darmstadt, Germany) (GM17). *L. casei* BL23, *L. plantarum* WCFS1, *L. fermentum* INIA 584L and L. *fermentum* INIA 832L were routinely cultivated at 37 °C in MRS broth (BD Biosciences, Le Pont de Claix, France), under anaerobic conditions (10% H_2, 10% CO_2 y 80% N_2. Whitley DG250 Anaerobic workstation, Don Whitley Scientific Limited, West Yorkshire, UK). *S. isoflavoniconvertens* DSM22006, used as equol and 5-hydroxy-equol positive control, was grown in Wilkins-Chalgren broth (Oxoid, Ltd. Basingstoke, England) at 37 °C under anaerobic conditions.

2.2. Cloning of genes involved in the transformation of daidzein to equol from S. isoflavoniconvertens

Based on the published genome of *S. isoflavoniconvertens* DSM 22006, specific primers (Table 2) were designed to amplify the genes *dzr*, *ddr*, *tdr* and *ifc*A. The genes *ddr* and *tdr* were amplified together in a single amplicon using the F-tdr and R-ddr oligonucleotides (Fig. 1). The forward F-tdr primer introduced a *BspH*I restriction site around the initiation codon of the THDR gene, and the reverse R-ddr primer introduced an *Xba*I site downstream of the stop codon of the DHDR gene. The PCR product was digested with the same restriction enzymes and ligated into the vector pNZ:TuR (Landete et al., 2015), previously digested with *Xba*I and *Nco*I, which produces *BspH*I compatible cohesive ends, to obtain the plasmid, pNZ:TuR.tdr.ddr.

The gene encoding DDRC (*ifc*A) was amplified by PCR using F-rac and R-rac primers (Table 2). Additionally, *ifc*A, was amplified together with the adjacent genes *ifc*B and *ifc*C, which are located downstream from *ifc*A and upstream of *tdr* in the *S. isoflavoniconvertens* genome (Fig. 1), by using the F-rac and R-ifcABC oligonucleotides. The forward primer F-rac introduced a *Pci*I site before the initiation codon of *ifc*A, and the reverse primers R-rac and R-ifcABC introduced an *Xba*I site downstream of the stop codon of *ifc*A and *ifc*C respectively. The PCR products were digested with the two restriction enzymes and ligated into the vector pNZ:TuR (Landete et al., 2015), previously digested with *Xba*I and *Nco*I, which produces *Pci*I compatible cohesive ends. The resulting plasmids were pNZ:TuR.ifcA and pNZ:TuR.ifcABC. Finally, joint cloning of *dzr*, *ddr* and *tdr* was attempted by using the primers F-tdr and R-DaidR.

The constructed plasmids were individually transformed into L. *lactis* MG1363 by electroporation (Landete et al., 2014); transformants were selected in GM17 agar with chloramphenicol (5 μg/mL, Merck KGaA) and checked by restriction mapping and sequencing the inserted fragment. *L. lactis* MG1363 was the host organism for subsequent transformations of L. *casei* BL23, *L. plantarum* WCFS1, *L. fermentum* INIA 584L

Table 1
Bacterial strains and plasmids used in this work.

Strains	Plasmid harbored	Reference
Lactococcus lactis subsp. *cremoris* MG1363		Gasson (1983)
L. lactis MG1363 pNZ:TuR	pNZ:TuR	Landete et al. (2015)
L. lactis MG1363 pNZ:TuR.dzr	pNZ:TuR.dzr	Peirotén et al. (2020b)
L. lactis MG1363 pNZ:TuR.tdr.ddr	pNZ:TuR.tdr.ddr	This work
L. lactis MG1363 pNZ:TuR.ifcA	pNZ:TuR.ifcA	This work
L. lactis MG1363 pNZ:TuR.ifcABC	pNZ:TuR.ifcABC	This work
Lacticaseibacillus casei BL23		Mazé et al., 2010
L. casei BL23 pNZ:TuR.dzr	pNZ:TuR.dzr	Peirotén et al. (2020b)
L. casei BL23 pNZ:TuR.tdr.ddr	pNZ:TuR.tdr.ddr	This work
L. casei BL23 pNZ:TuR.ifcA	pNZ:TuR.ifcA	This work
L. casei BL23 pNZ:TuR.ifcABC	pNZ:TuR.ifcABC	This work
Lactiplantibacillus plantarum WCFS1		Kleerebezem et al. (2003)
L. plantarum WCFS1 pNZ:TuR.dzr	pNZ:TuR.dzr	Peirotén et al. (2020b)
L. plantarum WCFS1 pNZ:TuR.tdr.ddr	pNZ:TuR.tdr.ddr	This work
L. plantarum WCFS1 pNZ:TuR.ifcA	pNZ:TuR.ifcA	This work
L. plantarum WCFS1 pNZ:TuR.ifcABC	pNZ:TuR.ifcABC	This work
Limosilactobacillus fermentum INIA 584 L		Peirotén et al. (2020b)
L. fermentum INIA 584L pNZ:TuR.dzr	pNZ:TuR.dzr	Peirotén et al. (2020b)
L. fermentum INIA 584L pNZ:TuR.tdr.ddr	pNZ:TuR.tdr.ddr	This work
L. fermentum INIA 584L pNZ:TuR.ifcA	pNZ:TuR.ifcA	This work
L. fermentum INIA 584L pNZ:TuR.ifcABC	pNZ:TuR.ifcABC	This work
Limosilactibacillus fermentum INIA 832 L		This work
L. fermentum INIA 832L pNZ:TuR.dzr	pNZ:TuR.dzr	This work
L. fermentum INIA 832L pNZ:TuR.tdr.ddr	pNZ:TuR.tdr.ddr	This work
L. fermentum INIA 832L pNZ:TuR.ifcA	pNZ:TuR.ifcA	This work
L. fermentum INIA 832L pNZ:TuR.ifcABC	pNZ:TuR.ifcABC	This work
Slackia isoflavoniconvertens DSM22006		Matthies et al. (2009)

Table 2
Plasmids used in this work.

	Sequence or description	References
Primers		This work
F-tdr	5′- TTTTCATGACAGAATTCGACGTTGAATACGATC	This work
R-ddr	5′- TTTTCTAGATTAGGCGATTTCGCCCTGCATAGCGC	This work
F-rac	5′- TTTACATGTTGCTCAAGGGCGAGTTTGCAGCATTGGC	This work
R-rac	5′- TTTTCTAGACTACTCAGCGTCCACGTCGCAAAC	This work
R-ifcABC	5′- TTTTCTAGATTACTTCAGAGCTTGAATAAATGCAGGGAG	This work
R-Daidr	5′- TTTTCTAGACTACACCATGCGCGCTACGGC	Peirotén et al. (2020b)
Plasmids		
pNZ:TuR	pNZ8048 with promoter of elongation factor Tu of *Lactobacillus reuteri* CECT925 replacing P_{nis}	Landete et al. (2015)
pNZ:TuR.dzr	pNZ:TuR with the daidzein reductase (DZNR) gene (*dzr*) from *S. isoflavoniconvertens* DSM 22006	Peirotén et al. (2020b)
pNZ:TuR.tdr.ddr	pNZ:TuR with the dihydrodaidzein reductase (DHDR) and tetrahydrodaidzein reductase (THDR) genes (*ddr* and *tdr* respectively) from *S. isoflavoniconvertens* DSM 22006	This work
pNZ:TuR.ifcA	pNZ:TuR with the dihydrodaidzein racemase (DDRC) gene (*ifc*A) from *S. isoflavoniconvertens* DSM 22006	This work
pNZ:TuR.ifcABC	pNZ:TuR with the dihydrodaidzein racemase (DDRC) gene (*ifc*A) and the adjacent genes *ifc*B and *ifc*C from *S. isoflavoniconvertens* DSM 22006	This work

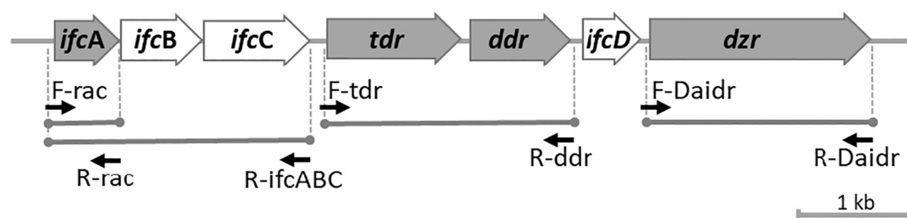

Fig. 1. Schematic representation of gene organization implicated in the equol production in *S. isoflavoniconvertens*. Primers for the amplification of *dzr*, *tdr* + *ddr*, *ifc*A, and *ifc*A + *ifc*B + *ifc*C genes are shown.

and L. *fermentum* INIA 832L with each of the plasmids (Table 1). These other LAB strains were transformed by electroporation followed by selection in MRS agar with chloramphenicol (5 μg/mL).

2.3. DHD and DHG metabolization by LAB harbouring pNZ:TuR.tdr.ddr, pNZ:TuR.ifcA and pNZ:TuR.ifcABC in culture medium

L. lactis MG1363, *L. casei* BL23, *L. plantarum* WCFS1, *L. fermentum* INIA 584L and *L. fermentum* INIA 832L, containing pNZ:TuR.tdr.ddr, as well as their respective parental strains, were added individually (1% *v*/v, between 1 × 10^7 and 1 × 10^8 cfu/mL) to BHI medium (Condalab, Torrejón de Ardoz, Spain), supplemented with DHD (50 mg/L; 195.12 μM) and DHG (50 mg/L, 183.65 μM) (Toronto Research Chemicals, Toronto, Canada). Incubation was performed at their optimal temperature (30 °C or 37 °C), for 72 h under anaerobic conditions.

Moreover, a series of co-cultures were performed in the same conditions described above, pairing couples of strains harbouring the genes of the reductases DHDR and THDR (pNZ:TuR.tdr.ddr) on one side, and the gene encoding DDRC (either the pNZ:TuR.ifcA or pNZ:TuR.ifcABC) on the other.

S. isoflavoniconvertens DSM22006 was grown in Wilkins-Chalgren broth supplemented with the same amounts of DHD and DHG, and was incubated at 37 °C under anaerobic conditions.

2.4. Daidzein and genistein metabolization by LAB harbouring pNZ:TuR.dzr, pNZ:TuR.tdr.ddr, pNZ:TuR.ifcA and pNZ:TuR.ifcABC in culture medium and cow's milk

Three different combinations of the transformants of each LAB were co-cultivated (1% *v*/v, between 1 × 10^7 and 1 × 10^8 cfu/mL) in BHI medium, supplemented with daidzein (50 mg/L; 196.77 μM) and genistein (50 mg/L; 185.02 μM) (LC Laboratories, Woburn, MA, USA) and incubated at their optimal temperature (30 °C or 37 °C), for 72 h under anaerobic conditions. Parental strains cultivated in the same conditions were used as controls. For each strain, different combinations were made of the strains harbouring i) *pNZ:TuR.dzr* (Peirotén et al., 2020b) and *pNZ:TuR.tdr.ddr;* ii) *pNZ:TuR.dzr*, *pNZ:TuR.tdr.ddr* and *pNZ:TuR.ifcA; iii) pNZ:TuR.dzr*, *pNZ:TuR.tdr.ddr* and *pNZ:TuR.ifcABC. S. isoflavoniconvertens* DSM22006 was grown in Wilkins-Chalgren broth supplemented with the same amounts of daidzein and genistein, and was incubated at 37 °C under anaerobic conditions.

Moreover, *L. fermentum* INIA 584L and *L. fermentum* INIA P832L were subjected to a similar incubation using cow's milk (reconstituted cow skim milk prepared according to the manufacturer's specifications, BD Biosciences) as matrix for the metabolization of daidzein and genistein, which were added in the same concentrations as before. The combinations used in this experiment for each of the strains were i) *pNZ:TuR.dzr* and *pNZ:TuR.tdr.ddr;* ii) *pNZ:TuR.dzr*, *pNZ:TuR.tdr.ddr* and *pNZ:TuR.ifcA*. Parental strains were again used as controls. Incubations were carried out for 72 h under anaerobic conditions at 37 °C.

2.5. Extraction of isoflavones

2.5.1. Extraction of isoflavones from culture medium

After incubations of the parental and transformed LAB in the combinations and conditions described above, isoflavones were extracted from the culture medium twice with 2 mL of diethyl ether and twice with 2 mL of ethyl acetate, according to Gaya et al. (2016a). The solvents were evaporated at room temperature under a N_2 stream, and the residue was dissolved in 300 μL methanol/water (50:50, *v*/v). Filtering, through a 0.22 μm cellulose acetate filter (Millipore, Madrid, Spain), was performed before transferring the extracts to HPLC vials and storing them at −20 °C.

2.5.2. Extraction of isoflavones from cow's milk

After incubation in cow's milk of the different combinations of LAB

described in Section 2.4., isoflavones were extracted following the official AOAC method (Collison et al., 2008). Briefly, 1 mL of the sample was mixed with 500 μL of acetonitrile and 150 μL of H_2O, shaken vigorously for 60 min and centrifuged for 10 min at 13000 rpm. The supernatant was filtered through a 0.22 μm PFTE membrane (Whatman; Cytiva, Little Chalfont, UK), and stored at −20 °C.

2.6. Identification and quantification of isoflavones

Isoflavones were analyzed by HPLC-ESI/MS as described in Gaya et al. (2016a) and Gaya et al. (2016b). Quantification was carried out by means of external standard calibration curves of equol and the precursors DHD, DHG, daidzein and genistein (LC Laboratories, Woburn, MA, USA). Stock solutions (10 mg/L) of polyphenols were prepared in DMSO (Merck KGaA). 5-hydroxy-equol was not available as a standard in any commercial house. The incubation of genistein and DHG with *S. isoflavoniconvertens* DSM22006 allowed us to identify the 5-hydroxy-equol peak by HPLC-ESI/MS analysis. So, 5-hydroxy-equol was quantified using the calibration curve of the more similar compound, equol.

2.7. Statistical analysis

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

3. Results

3.1. Equol and 5-hydroxy-equol production from DHD and DHG by recombinant LAB strains in culture medium

The parental strains *L. lactis* MG1363, *L. casei* BL23, *L. plantarum* WCFS1, *L. fermentum* INIA 584L and L. *fermentum* INIA 832L were unable to produce equol or 5-hydroxy-equol from DHD and DHG.

All the LAB strains harbouring pNZ:TuR.tdr.ddr, encoding DHDR and THDR, were able to produce equol from DHD (Table 3). Nevertheless, the yields in equol production varied greatly between strains. The two *L. fermentum* strains consumed DHD in similar levels to *S. isoflavoniconvertens* and showed the highest production among the recombinant LAB strains, transforming nearly half of the DHD added to the medium into equol, although they did not reach the levels of production of *S. isoflavoniconvertens*. The rest of LAB harbouring pNZ:TuR.tdr.ddr showed lower metabolization of DHD and smaller amounts of equol produced. On its part, 5-hydroxy-equol was only produced by the *L. fermentum* strains and with lower yields than the ones obtained with equol. Interestingly, although the two *L. fermentum* strains harbouring pNZ:TuR.tdr.ddr consumed more DHG than *S. isoflavoniconvertens*, this had no reflection in more 5-hydroxy-equol production.

The co-incubation of the LAB strains harbouring pNZ:TuR.tdr.ddr with the correspondent strains containing DDRC, harbouring either pNZ:TuR.ifcA or pNZ:TuR.ifcABC, did not result in significant changes in the equol and 5-hydroxy-equol production from DHD and DHG in BHI medium (Table 3).

Table 3
Production of equol and 5-hydroxy-equol from DHD (195.12 μM) and DHG (183.65 μM) by parental and transformed strains of LAB and *S. isoflavoniconvertens* DSM22006 in culture medium.

Strains	DHD (μM)	Equol (μM)	DHG (μM)	5-Hydroxy-equol (μM)
L. lactis MG1363				
Parental strain (Control)	180.54 ± 5.82 [de]	n.d.[a]	177.71 ± 1.80 [def]	n.d.[a]
ddr-tdr	131.50 ± 15.03 [b]	46.77 ± 1.57 [e]	160.37 ± 7.62 [bcd]	n.d.[a]
ddr-tdr + ifcA	141.44 ± 2.98 [b]	34.68 ± 6.29 [de]	168.33 ± 2.21 [cdef]	n.d.[a]
ddr-tdr + ifcABC	143.21 ± 0.72 [bc]	35.42 ± 2.31 [de]	162.86 ± 3.65 [bcde]	n.d.[a]
L. casei BL23				
Parental strain (Control)	178.28 ± 15.47 [cde]	n.d.[a]	181.23 ± 0.94 [ef]	n.d.[a]
ddr-tdr	145.73 ± 0.71 [bcd]	31.77 ± 1.57 [cde]	163.27 ± 1.52 [bcde]	n.d.[a]
ddr-tdr + ifcA	142.81 ± 6.70 [bc]	21.68 ± 6.29 [bcd]	164.02 ± 3.44 [bcde]	n.d.[a]
ddr-tdr + ifcABC	140.98 ± 2.83 [b]	22.44 ± 5.17 [bcd]	160.23 ± 2.05 [bcd]	n.d.[a]
L. plantarum WCFS1				
Parental strain (Control)	177.21 ± 0.94 [cde]	n.d.[a]	175.41 ± 2.17 [def]	n.d.[a]
ddr-tdr	144.95 ± 12.34 [bc]	22.14 ± 1.56 [bcd]	165.27 ± 4.34 [cdef]	n.d.[a]
ddr-tdr + ifcA	136.76 ± 4.24 [b]	11.64 ± 6.29 [ab]	154.29 ± 1.81 [bc]	n.d.[a]
ddr-tdr + ifcABC	139.87 ± 1.41 [b]	13.23 ± 7.13 [abc]	169.41 ± 0.90 [cdef]	n.d.[a]
L. fermentum INIA 584L				
Parental strain (Control)	186.17 ± 2.91 [e]	n.d.[a]	179.84 ± 8.37 [ef]	n.d.[a]
ddr-tdr	60.48 ± 15.98 [a]	93.13 ± 2.89 [f]	109.27 ± 6.29 [a]	22.06 ± 1.53 [cd]
ddr-tdr + ifcA	54.65 ± 6.14 [a]	87.72 ± 4.53 [f]	116.87 ± 5.75 [a]	16.37 ± 3.32 [bc]
ddr-tdr + ifcABC	55.05 ± 1.80 [a]	86.34 ± 2.84 [f]	119.55 ± 2.81 [a]	17.12 ± 3.28 [bc]
L. fermentum INIA 832L				
Parental strain (Control)	184.98 ± 3.10 [e]	n.d.[a]	183.29 ± 4.16 [f]	n.d.[a]
ddr-tdr	72.54 ± 7.65 [a]	89.33 ± 5.79 [f]	104.18 ± 2.90 [a]	19.42 ± 0.73 [bcd]
ddr-tdr + ifcA	51.76 ± 4.33 [a]	81.70 ± 3.28 [f]	114.33 ± 1.26 [a]	17.56 ± 2.80 [bc]
ddr-tdr + ifcABC	53.78 ± 2.74 [a]	84.11 ± 1.14 [f]	119.73 ± 3.54 [a]	14.28 ± 3.32 [b]
S. isoflavoniconvertens DSM22006	54.78 ± 1.58 [a]	122.55 ± 7.72 [g]	145.34 ± 2.01 [b]	25.90 ± 1.45 [d]

[a–g]Values in the same column with different superscript differ significantly ($P < 0.01$); n.d. not detected.

3.2. Equol and 5-hydroxy-equol production from daidzein and genistein in culture medium by recombinant LAB strains

After corroborating the transformation of DHD and DHG by LAB strains harbouring pNZ:TuR.tdr.ddr, new co-incubations with daidzein and genistein as precursors were carried out. In this case, the co-cultures included the strains harbouring pNZ:TuR.dzr, which in previous works had shown to reduce daidzein and genistein into DHD and DHG (Peirotén et al., 2020b). All the strains combinations, i.e. harbouring the *dzr* gene and the combined *ddr* and *tdr* genes, resulted in an efficient transformation of daidzein into DHD and of genistein into DHG, and in the production of equol (Table 4). Once more, the combinations of the recombinant *L. fermentum* strains showed the highest production of equol and were the only ones producing 5-hydroxy-equol. Conversely, the correspondent parental strains showed only small amounts of DHD and no production of equol, DHG or 5-hydroxy-equol.

Once the production of equol and 5-hydroxy-equol was demonstrated by the heterologous expression of the genes *dzr*, *ddr* and *tdr* in *L. fermentum* strains, we worked on the joint cloning of *ddr*, *tdr* and *dzr* with primers F-tdr and R-Daidr (Fig. 1). However, although we achieved amplification, we did not obtain transformants and thus, the transformation of daidzein and genistein by sole strains expressing simultaneously the three genes could not be tested.

The addition of the DDRC to the co-cultures, i.e. incubation of triplets of strains harbouring pNZ:TuR.dzr, pNZ:TuR.tdr.ddr and pNZ:TuR.ifcA

Table 4
Metabolism of daidzein (196.77 μM) and genistein (185.02 μM) by parental and transformed LAB strains and *S. isoflavoniconvertens* DSM22006 in culture medium.

Strains	Daidzein (μM)	DHD (μM)	Equol (μM)	Genistein (μM)	DHG (μM)	5-Hydroxy-equol (μM)
L. lactis MG1363						
Parental strain (control)	182.03 ± 3.51 [bc]	2.36 ± 0.66 [a]	n.d.[a]	175.66 ± 3.17 [c]	n.d.[a]	n.d.[a]
dzr + ddr-tdr	12.00 ± 0.26 [a]	66.90 ± 2.66 [b]	24.96 ± 2.55 [bc]	4.38 ± 0.24 [a]	140.49 ± 7.92 [bc]	n.d.[a]
dzr + ddr-tdr + ifcA	14.91 ± 0.14 [a]	91.92 ± 3.20 [cde]	17.35 ± 2.46 [b]	4.77 ± 1.56 [a]	127.43 ± 5.10 [b]	n.d.[a]
dzr + ddr-tdr + ifcABC	13.49 ± 1.24 [a]	87.12 ± 1.80 [cd]	18.26 ± 0.78 [b]	4.59 ± 0.86 [a]	128.33 ± 4.10 [b]	n.d.[a]
L. casei BL23						
Parental strain (control)	179.27 ± 1.70 [bc]	0.04 ± 0.00 [a]	n.d.[a]	178.46 ± 4.10 [c]	n.d [a.]	n.d.[a]
dzr + ddr-tdr	13.23 ± 2.76 [a]	95.85 ± 6.46 [de]	16.12 ± 2.18 [ab]	11.12 ± 2.08 [a]	133.59 ± 7.32 [b]	n.d.[a]
dzr + ddr-tdr + ifcA	22.67 ± 0.85 [a]	81.07 ± 4.22 [bcd]	13.36 ± 2.36 [ab]	9.70 ± 0.65 [a]	142.63 ± 2.90 [bc]	n.d.[a]
dzr + ddr-tdr + ifcABC	18.11 ± 1.55 [a]	85.96 ± 1.46 [cd]	12.35 ± 1.76 [ab]	10.12 ± 3.26 [a]	131.22 ± 7.86 [b]	n.d.[a]
L. plantarum WCFS1						
Parental strain (control)	167.52 ± 2.83 [b]	1.76 ± 0.85 [a]	n.d.[a]	165.61 ± 5.47 [bc]	n.d.[a]	n.d.[a]
dzr + ddr-tdr	12.54 ± 0.26 [a]	90.12 ± 6.63 [cde]	18.23 ± 1.56 [b]	11.23 ± 2.06 [a]	131.34 ± 4.55 [b]	n.d.[a]
dzr + ddr-tdr + ifcA	13.26 ± 4.65 [a]	78.43 ± 3.21 [bc]	11.35 ± 0.85 [ab]	8.17 ± 1.77 [a]	129.45 ± 5.10 [b]	n.d.[a]
dzr + ddr-tdr + ifcABC	19.06 ± 4.37 [a]	87.13 ± 8.25 [cd]	17.35 ± 3.41 [b]	9.74 ± 1.53 [a]	132.70 ± 9.40 [b]	n.d.[a]
L. fermentum INIA 584L						
Parental strain (control)	171.45 ± 4.84 [bc]	1.97 ± 0.06 [a]	n.d.[a]	159.66 ± 6.47 [b]	n.d.[a]	n.d.[a]
dzr + ddr-tdr	13.51 ± 0.82 [a]	114.18 ± 4.11 [f]	47.15 ± 7.35 [d]	2.43 ± 1.54 [a]	128.00 ± 5.54 [b]	9.26 ± 1.15 [bc]
dzr + ddr-tdr + ifcA	9.89 ± 0.48 [a]	1.98 ± 0.78 [a]	169.72 ± 8.40 [f]	7.20 ± 2.68 [a]	135.27 ± 5.71 [bc]	14.80 ± 1.40 [d]
dzr + ddr-tdr + ifcABC	10.67 ± 2.52 [a]	2.48 ± 0.91 [a]	172.70 ± 7.35 [f]	7.80 ± 1.45 [a]	130.17 ± 2.43 [b]	13.70 ± 1.24 [cd]
L. fermentum INIA 832L						
Parental strain (control)	178.33 ± 5.82 [bc]	5.55 ± 1.96 [a]	n.d.[a]	159.66 ± 6.48 [b]	n.d.[a]	n.d.[a]
dzr + ddr-tdr	15.33 ± 1.02 [a]	107.13 ± 4.76 [ef]	41.89 ± 2.31 [cd]	7.61 ± 1.50 [a]	136.27 ± 1.60 [bc]	8.12 ± 2.65 [b]
dzr + ddr-tdr + ifcA	12.67 ± 3.58 [a]	15.08 ± 3.18 [a]	156.62 ± 2.32 [f]	11.33 ± 1.67 [a]	150.95 ± 6.36 [bc]	12.76 ± 2.83 [bcd]
dzr + ddr-tdr + ifcABC	12.73 ± 2.00 [a]	10.62 ± 2.11 [a]	164.28 ± 5.10 [f]	7.21 ± 2.68 [a]	142.17 ± 7.07 [bc]	12.09 ± 1.22 [bcd]
S. isoflavoniconvertens DSM22006	19.73 ± 4.24 [a]	88.28 ± 1.35 [cd]	76.70 ± 2.14 [e]	3.70 ± 0.06 [a]	159.03 ± 6.01 [c]	14.76 ± 0.32 [d]

[a-f]Values in the same column with different superscript differ significantly ($P < 0.01$); n.d. not detected.

or pNZ:TuR.ifcABC, caused and increment on the equol production in the two *L. fermentum* strains, which showed a high level of DHD transformation into this metabolite, reaching an 80–87% of transformation of the daidzein into equol and surpassing the yields of equol production by *S. isoflavoniconvertens* DSM 22006 (Table 4). A similar tendency was observed regarding the production of 5-hydroxy-equol, which showed a slight increase in the two *L. fermentum* strains when *ifc*A was present. In the rest of recombinant LAB there was no significant changes, except for a slight increase of DHD in the case of L. *lactis* MG1363 when strains harbouring pNZ:TuR.ifcA or pNZ:TuR.ifcABC were added to the medium.

3.3. *Equol and 5-hydroxy-equol production by L. fermentum strains in cow's milk supplemented with daidzein and genistein*

In order to seek the applicability of the recombinant LAB strains in the development of functional foods enriched in equol, the two *L. fermentum* strains were incubated in cow's milk supplemented with daidzein and genistein.

The different combinations of recombinant strains showed similar behavior to that observed in culture medium, showing high metabolization of daidzein and genistein and the production of equol and 5-hydroxy-equol in the supplemented milk (Table 5). For those strains, the addition of the strain harbouring pNZ:TuR.ifcA to the mixture of recombinant strains resulted in a clear increase of the equol yields, with around four times more transformation of the initial daidzein into equol.

4. Discussion

It has been widely demonstrated that LAB strains are able to deglycosylate glycoside isoflavones in culture media and beverages (Gaya et al., 2016c; Rekha and Vijayalakshmi, 2011). Even, some LAB strains are capable of producing high concentrations of daidzein and genistein in soy beverages (Delgado et al., 2019; Peirotén et al., 2020a). However, equol production has been mainly described within the family *Eggerthellaceae* (Mayo et al., 2019), while LAB strains completing the transformation of daidzein and genistein are scarce, and those described (Heng et al., 2019; Uchiyama et al., 2007) belong to species without a tradition of usage in food fermentations and not included in the GRAS and QPS lists. The heterologous production of equol and 5-hydroxy-equol has been previously explored by means of recombinant *E. coli* strains expressing the enzymes involved in the metabolic transformation of daidzein (Kawada et al., 2016; Lee et al., 2016). In this work, we have explored the viability of heterologous production of equol by different

Table 5
Metabolism of daidzein (196.77 μM) and genistein (185.02 μM) by parental and transformed strains of L. *fermentum* INIA 584 L and *L. fermentum* INIA 832 L in cow's milk.

Strains	Daidzein (μM)	DHD (μM)	Equol (μM)	Genistein (μM)	DHG (μM)	5-Hydroxy-equol (μM)
L. fermentum INIA 584L						
Parental strain (control)	177.68 ± 5.85 [b]	5.94 ± 0.41 [a]	n.d.[a]	170.38 ± 2.74 [b]	11.13 ± 0.29 [a]	n.d.[a]
dzr + ddr-tdr	16.34 ± 1.22 [a]	52.48 ± 4.46 [b]	35.57 ± 2.20 [b]	10.34 ± 2.01 [a]	148.47 ± 1.33 [d]	3.41 ± 1.98 [a]
dzr + ddr-tdr + ifcA	7.73 ± 1.68 [a]	13.18 ± 2.95 [a]	120.94 ± 11.5 [c]	2.53 ± 0.77 [a]	125.23 ± 6.63 [bc]	5.40 ± 0.45 [a]
L. fermentum INIA 832L						
Parental strain (control)	181.56 ± 57.35 [b]	4.08 ± 1.16 [a]	n.d.[a]	172.17 ± 1.82 [b]	4.88 ± 0.47 [a]	n.d.[a]
dzr + ddr-tdr	18.22 ± 4.66 [a]	59.33 ± 7.22 [b]	31.17 ± 1.10 [ab]	9.26 ± 2.44 [a]	140.74 ± 2.30 [cd]	3.18 ± 1.98 [a]
dzr + ddr-tdr + ifcA	6.94 ± 2.15 [a]	11.76 ± 1.82 [a]	109.94 ± 9.65 [c]	3.52 ± 1.87 [a]	117.98 ± 5.35 [b]	4.30 ± 0.47 [a]

[a-d]Values in the same column with different superscript differ significantly ($P < 0.01$); n.d. not detected.

LAB belonging to species commonly used in food fermentation and thus GRAS.

The joint cloning of *ddr* and *tdr* from *S. isoflavoniconvertens* allowed the transformation of DHD into equol by recombinant LAB belonging to the *L. lactis*, *L. casei*, *L. plantarum* and *L. fermentum* species (Table 3), while the addition of *dzr* resulted in the production of equol from daidzein (Table 4) in agreement with that described in recombinant *E. coli* (Tsuji et al., 2012). Interestingly, the two recombinant *L. fermentum* strains showed production, both from DHG and genistein, of 5-hydroxy-equol, whose production by recombinant *E. coli* has been scarcely reported (Lee et al., 2017). In addition, it was observed how the combination of DHDR and THDR led to a less efficient transformation of DHG than DHD and, therefore, the 5-hydroxy-equol production was much lower than the equol production. These results are in accordance with the lack of production of 5-hydroxy-equol by recombinant *E. coli* (Schröder et al., 2013) and the less efficient formation of 5-hydroxy-equol in comparison to equol observed in vitro and in vivo with intact cells of *S. isoflavoniconvertens* (Matthies et al., 2009).

The addition of the strains harbouring *ifc*A resulted in an increment of equol production from daidzein only in the case of the two *L. fermentum* strains. Similarly to the results of Shimada et al. (2012), the addition of the recombinant DDRC resulted in a 3.6 to 3.9 fold increase of equol production by the *L. fermentum* strains. The lack of activity of DDRC in the rest of the LAB strains tested, reflected in no increment of equol production, could be related to a lower activity of the enzymes DHDR and/or THDR in those strains, since they also produce less equol than the *L. fermentum* strains (Table 4), although other factors may be affecting. The apparent lack of activity of DDRC in *L. fermentum* when incubated with DHD is likely due to this commercial compound being a racemic mixture, while the DHD produced by DZNR from daidzein has been described to be mainly R-DHD (Shimada et al., 2012). Given also the described affinity of DHDR for S-DHD, the action of this racemase would be of importance when DHD is produced mainly as the R enantiomer, but not when using a racemic mixture as substrate. The importance of DDRC for an efficient production of 5-hydroxyequol has been also described (Lee et al., 2017). In this work, we found a slight increase on 5-hydroxy-equol production from genistein by the two L. *fermentum* strains upon the addition of the strains with the recombinant DDRC, although not statistically significative. Conversely, *S. isoflavoniconvertens* showed fewer production of equol from daidzein compared to DHD as precursor, achieving lower equol concentrations than the combination of L. *fermentum* strains harbouring *ifc*A (Table 4). In this regard, since both DZNR and DHDR need NADPH as cofactor, the transformation of daidzein could be interfering with the transformation of DHD, as compared with the transformation for DHD directly in which the NADPH is used only by DHDR. This could have a higher impact on *S. isoflavoniconvertens* since the reactions take place within the same cell, while the reactions are compartmentalized in *L. fermentum* strains, i.e. daidzein is reduced by *L. fermentum* pNZ:TuR.dzr while DHD is transformed by *L. fermentum* pNZ:TuR.tdr.ddr. This beneficial effect of compartmentalization in equol production has been described previously in recombinant *E. coli* (Lee et al., 2017).

The genes *ifc*B and *ifc*C, which are located between the genes *ifc*A and *tdr* in the *S. isoflavoniconvertens* cluster, were tested together with *ifc*A, resulting in no differences between adding the strains with just *ifc*A (pNZ:TuR.ifcA) or with the three genes (pNZ:TuR.ifcABC). This suggests that those two genes seem to have no influence in the equol production under the conditions tested.

S. isoflavoniconvertens produced higher concentration of equol and 5-hydroxy-equol than the majority or the different co-cultures of transformed LAB strains incubated with isoflavone precursors in medium (Tables 3 and 4). However, *L. fermentum* INIA 584L and L. *fermentum* INIA 832L harbouring the combination of *dzr*, *ifc*A, *tdr* and *ddr* surpassed the production of equol from daidzein showed by *S. isoflavoniconvertens* DSM22006, and equaled its production of 5-hydroxy-equol from genistein (Table 4).

Taking into account the equol production by the *L. fermentum* strains, we assayed the production of equol in a food matrix. We chose a food matrix with no isoflavones in its composition (cow's milk), and supplemented it with known amounts of daidzein and genistein. Similarly to the results with culture medium, the combination of the four genes, *dzr*, *ifc*A, *tdr* and *ddr*, resulted in a highly efficient production of equol in the incubated milk (Table 5). The use of soy beverage as base for this kind of product should take into account that it has a complex composition of isoflavones and that they are present mainly in the form of glycosides. Thus, in order to obtain an efficient production of equol, the glycosidase activity should be ensured in the form of another bacterial strain with high glycosidase activity or of a recombinant strain harbouring an efficient β-glucosidase (Gaya et al., 2020).

LAB strains expressing genes of interest have great potential in the development of functional products, such as food enriched in equol. Nevertheless, safety and legal issues must be taken into account in order to obtain an authorization, which, in the EU follows a case-by-case approach (EFSA, 2011) that has put the use of this kind of microorganisms under a de facto moratorium. Within this scenario, the products with transformed bacteria undergo less restrictive requirements if the bacteria and its DNA is not present in the final product. Seeing this, the more feasible alternative for a soy beverage enriched in equol and 5-hydroxy-equol by the fermentation of recombinant LAB strains would have to incorporate a later treatment to eliminate the bacteria and their DNA.

5. Conclusions

This is the first report concerning the use of recombinant bacteria with interest in food, such as LAB belonging to GRAS species, to produce equol and 5-hydroxy-equol. The combination of *L. fermentum* strains harbouring the *S. isoflavoniconvertens* enzymes DZNR, DDRC, DHDR and THDR showed efficient transformation of daidzein to equol, both in culture medium and in milk. This opens the possibility for the development of fermented foods enriched in equol.

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Declaration of competing interest

The authors declare no conflict of interest.

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