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 (dar), dihydrodaidzein reductase (ddr), tetrahydrodaidzein reductase (tdr) and dihydrodaidzein racemase (ifcA) genes, from Slackia isoflavoniconvertens DSM22006, were cloned into the vector pNZ:TuR, under a strong constitutive promoter (TuR). Lactococcus lactis MG1363, Lactocaseibacillus 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 metabolism of daidzein and genistein by the combination of strains harbouring pNZ: TuR.ddr 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 (Aguirar et al., 2007). They are classified as phytoestrogens since their structures resemble that of estrogen and have weak affinity for the estrogen receptor (Yaya 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 DHG 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 derive 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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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 Enterobacteriaceae, such as Adlerecreus equolifaciens, Assacharobacter bacterius, Eggerthella spp., Enterorhabdus mucosolavus, Slackia isoflavoniconvertens and Slackia equolifaciens (Mao et al., 2019). Besides these species, some intestinal lactose acid bacteria (LAB) strains have been described as equol-producers, i.e. Lactobacillus intestinatis (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 Lactobacillus fermentum, Lacticaseibacillus casei and Lactobacillus plantarum, 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 activity transforming the enantiomers favors the equol production (Shi et al., 2020). The production of equol and 5-hydroxy-equol from different pre-cursors in culture medium and in a food matrix was studied.

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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% H2, 10% CO2 y 80% N2). Whitley DG250 Anaerobic workstation, Don Whitley Scientific Limited, West Yorkshire, UK). S. isoflavoniconvertens DSM22006, used as equal 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 ddr, ddr, tdr and ifc. The genes ddr and tdr were amplified together in a single ampiclon using the F-ddr and R-ddr oligonucleotides (Fig. 1). The forward F-ddr primer introduced a BspHI restriction site around the initiation codon of the THDR gene, and the reverse R-ddr primer introduced an XbaI 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 XbaI and NcoI, which produces BspHI compatible cohesive ends, to obtain the plasmid, pNZ:TuR.tdr.ddr.

The gene encoding DDRC (ifcA) was amplified by PCR using F-rac and R-rac primers (Table 2). Additionally, ifcA was amplified together with the adjacent genes ifcB and ifcC, which are located downstream from ifcA 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 site before the initiation codon of ifcA, and the reverse primer R-rac and R-ifcABC introduced an XbaI site downstream of the stop codon of ifcA and ifcC 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 XbaI and NcoI, which produces PciI compatible cohesive ends. The resulting plasmids were pNZ:TuR.ifcA and pNZ:TuR.ifcABC. Finally, joint cloning of ddr and tdr 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.
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 metabolism by LAB harbouring pNZ:TuR.tdr.ddr, pNZ:TuR.dzr 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 BH 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 adjacent genes ifcA and ifcC from S. isoflavoniconvertens DSM 22006 (from Ardoz, Spain), supplemented with DHD (50 mg/L; 195.12 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; ii) pNZ:TuR.tdr.ddr; ii) pNZ:TuR.dzr, pNZ:TuR.tdr.ddr and pNZ:TuR.ifcABC. S. isoflavoniconvertens DSM22006 was grown in Wilkins-Chalgen broths 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 skin milk prepared according to the manufacturer’s specifications, BD Biosciences) as matrix for the metabolism 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.ifcABC. S. isoflavoniconvertens DSM22006 was grown in Wilkins-Chalgen broth supplemented with the same amounts of daidzein and genistein, and was incubated at 37 °C under anaerobic conditions.

### 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 N2 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 H2O, 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 recombinant LAB strains, transforming nearly half of the DHD added to BHI medium (Table 3).

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 WCF51, 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 equal production varied greatly between strains. The two L. fermentum strains consumed DHD in similar levels to S. isoflavoniconvertens and showed the highest production from 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 DDCR, 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).

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.dzzr, which in previous works had shown to reduce daidzein and genistein into DHD and DHG (Peiróten et al., 2020b). All the strains combinations, i.e. harbouring the dzzr gene and the combined tdr 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 dzzr, ddr and tdr in L. fermentum strains, we worked on the joint cloning of ddr, tdr and dzzr 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 DDCR to the co-cultures, i.e. incubation of triplets of strains harbouring pNZ:TuR.dzzr, pNZ:TuR.tdr.ddr and pNZ:TuR.ifcA

| Table 3 | Production of equol and 5-hydroxy-equol from DHD (195.1 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| 180.54 ± 5.92 ±                | n.d. ±                        | 177.71 ± 1.85 ±              | n.d. ±                       |
| Parental strain (Control) | 131.50 ± 15.03 ±              | 46.77 ± 1.57 ±               | 160.37 ± 7.62 ±             | n.d. ±                       |
| ddr-tdr         | 141.44 ± 2.98 ±              | 34.68 ± 6.29 ±               | 168.33 ± 2.21 ±             | n.d. ±                       |
| ddr-tdr + ifcA  | 142.21 ± 0.72 ±              | 35.42 ± 2.31 ±               | 162.86 ± 3.65 ±             | n.d. ±                       |
| ddr-tdr + ifcABC| 1.40 ± 2.83 ±                | 22.44 ± 5.17 ±               | 160.23 ± 2.05 ±             | n.d. ±                       |
| L. casei BL23   | 172.88 ± 15.47 ±             | n.d. ±, ±                     | 181.23 ± 0.94 ±             | n.d. ±                       |
| Parental strain (Control) | 145.73 ± 0.71 ±              | 31.77 ± 1.57 ±               | 163.27 ± 1.52 ±             | n.d. ±                       |
| ddr-tdr         | 142.81 ± 6.70 ±              | 21.68 ± 6.29 ±               | 164.02 ± 3.44 ±             | n.d. ±                       |
| ddr-tdr + ifcA  | 140.98 ± 1.41 ±              | 22.44 ± 7.13 ±               | 160.23 ± 9.06 ±             | n.d. ±                       |
| L. plantarum WCF51| 177.21 ± 0.94 ±             | n.d. ±, ±                     | 175.41 ± 2.17 ±             | n.d. ±                       |
| Parental strain (Control) | 144.95 ± 12.34 ±              | 22.14 ± 1.56 ±               | 165.27 ± 4.33 ±             | n.d. ±                       |
| ddr-tdr         | 136.76 ± 4.24 ±              | 11.64 ± 6.29 ±               | 154.29 ± 1.81 ±             | n.d. ±                       |
| ddr-tdr + ifcA  | 139.87 ± 1.41 ±              | 13.23 ± 7.13 ±               | 169.41 ± 9.60 ±             | n.d. ±                       |
| L. fermentum INIA 584L | 186.17 ± 2.91 ±             | n.d. ±, ±                     | 179.84 ± 8.37 ±             | n.d. ±                       |
| Parental strain (Control) | 60.48 ± 15.98 ±              | 93.13 ± 2.89 ±               | 109.27 ± 6.29 ±             | 22.06 ± 1.53 ±               |
| ddr-tdr         | 54.65 ± 6.14 ±              | 87.72 ± 4.53 ±               | 116.87 ± 5.75 ±             | 16.37 ± 3.32 ±               |
| ddr-tdr + ifcA  | 55.05 ± 1.80 ±              | 86.34 ± 2.84 ±               | 119.55 ± 2.81 ±             | 17.12 ± 3.28 ±               |
| L. fermentum INIA 832L | 184.98 ± 3.10 ±             | n.d. ±, ±                     | 183.29 ± 4.16 ±             | n.d. ±                       |
| Parental strain (Control) | 72.54 ± 5.76 ±              | 89.33 ± 7.10 ±               | 104.18 ± 2.90 ±             | 19.42 ± 0.73 ±               |
| ddr-tdr         | 51.76 ± 4.33 ±              | 81.70 ± 3.28 ±               | 114.33 ± 1.26 ±             | 17.56 ± 2.80 ±               |
| ddr-tdr + ifcA  | 53.78 ± 2.74 ±              | 84.11 ± 1.14 ±               | 119.73 ± 3.54 ±             | 14.28 ± 3.32 ±               |
| S. isoflavoniconvertens DSM22006 | 54.78 ± 1.58 ±             | 122.55 ± 7.72 ±              | 145.34 ± 2.01 ±             | 25.90 ± 1.45 ±               |

Values in the columns with different superscript differ significantly (P < 0.01); n.d. not detected.
or pNZ:TuRIfcABC, caused and increased on the equal 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 equal and surpassing the yields of equal 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 ifcA 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:TuRIfcA or pNZ:TuRIfcABC were added to the medium.

3.3. Equal 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 equal, 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 equal and 5-hydroxy-equol in the supplemented milk (Table 5). For those strains, the addition of the strain harbouring pNZ:TuRIfcABC to the mixture of recombinant strains resulted in a clear increase of the equal yields, with around four times more transformation of the initial daidzein into equal.

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; Peiró et al., 2020a). However, equal production has been mainly described within the family Enterobacteriaceae (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 equal 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 equal by different

### Table 4

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<th>Strains</th>
<th>Daidzein (µM)</th>
<th>DHD (µM)</th>
<th>Equol (µM)</th>
<th>Genistein (µM)</th>
<th>DHG (µM)</th>
<th>5-Hydroxy-equol (µM)</th>
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<td>171.45 ± 4.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>165.61 ± 5.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>170.38 ± 2.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>177.68 ± 2.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>175.66 ± 1.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>171.45 ± 4.84&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>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. isoflavonicvertens* 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 dar resulted in the production of equol from daidzein (Table 4) in agreement with that described in recombinant *E. coli* (Tuji 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 DDHR and THDR led to a less efficient transformation of DHD 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* (Schroder 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. isoflavonicvertens* (Matthies et al., 2009).

The addition of the strains harbouring ifcA 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. isoflavonicvertens* showed fewer production of equol from daidzein compared to DHD as precursor, achieving lower equol concentrations than the combination of **L. fermentum** strains harbouring ifcA (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. isoflavonicvertens* 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.dar 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 ifcB and ifcC, which are located between the genes ifcA and tdr in the **S. isoflavonicvertens** cluster, were tested together with ifcA, resulting in no differences between adding the strains with just ifcA (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. isoflavonicvertens* 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 dar, ifcA, tdr and ddr surpassed the production of equol from daidzein showed by **S. isoflavonicvertens** DSM22006, and equaled its production of 5-hydroxy-equol from genistein (Table 4).

Taking into account the equal 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, dar, ifcA, 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. isoflavonicvertens** 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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