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Heterologous expression of equol biosynthesis genes from Adlercreutzia equolifaciens

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One sentence summary: Heterologous equol production from synthetic genes based on those of Adlercreutzia equolifaciens.

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ABSTRACT

Equol is the isoflavone-derived metabolite with the greatest estrogenic and antioxidant activity. It is produced from daidzein by fastidious and oxygen-susceptible intestinal bacteria, which hinders their use at an industrial scale. Therefore, expressing the equol production machinery into easily-cultivable hosts would expedite the heterologous production of this compound. In this work, four genes (racemase, tdr, ddr and dzr) coding for key enzymes involved in equol production in Adlercreutzia equolifaciens DSM19450T were synthesized and cloned in a pUC-derived vector (pUC57-equol) that was introduced in Escherichia coli. Recombinant clones of E. coli produced equol in cultures supplemented with daidzein (equol precursor) and dihydrodaidzein (intermediate compound). To check whether equol genes were expressed in Gram-positive bacteria, the pUC57-equol construct was cloned into the low-copy-number vector pIL252, and the new construct (pIL252-pUC57-equol) introduced into model strains of Lacticaseibacillus casei and Lactococcus lactis. L. casei clones carrying pIL252-pUC57-equol produced a small amount of equol from dihydrodaidzein but not from daidzein, while L. lactis recombinant clones produced no equol from either of the substrates. This is the first time that Adlercreutzia equolifaciens genes have been cloned and expressed in heterologous hosts. E. coli clones harboring pUC57-equol could be used for biotechnological production of equol.

Keywords: soy isoflavones; daidzein; equol; Adlercreutzia equolifaciens; daidzein reductase; dihydrodaidzein reductase; tetrahydrodaidzein reductase; gene cloning; gene expression

INTRODUCTION

Epidemiological and interventional studies suggest that consumption of soy and soy products helps to prevent, and can be used to treat, postmenopausal symptoms, cardiovascular and neurological diseases, osteoporosis and hormone-dependent cancers (Smeriglio et al. 2019; Zaheer and Humayoun Akhtar 2017). The beneficial effects of soy are attributed to the isoflavones and their bacteria-derived metabolites. Chemically, some of these compounds resemble the endogenous 17β-estradiol and possess hormone-like activity (Vitale et al. 2013; Franke, Lai and Halm 2014). Among the isoflavone-derived metabolites, equol, formed from daidzein in the human intestine by a few bacterial species, is the isoflavone metabolite...
with the strongest estrogenic and antioxidant activity (Mayo, Vázquez and Flórez 2019). However, it is produced in just 25–50% of people, depending on the human population to which they belong and their dietary habits. The persons carrying in their gut microbiota equol-producing species might be the only ones to fully benefit from isoflavone consumption (Birru et al. 2016).

Several equol-producing bacterial strains from the human gut have been identified and characterized over the last decades (Mayo, Vázquez and Flórez 2019). Most belong to minority populations of strict anaerobic species within the family Egerthellaaceae (phylum Actinobacteria) (Salam et al. 2020), including Adlercreuzia equolifaciens, Slackia isoflavoniconvertens and Slackia equiliciakensis (Vázquez et al. 2017; Braune and Blaut 2018). Equol biosynthesis in these bacteria proceeds via dihydrodaidzein and tetrahydrodaidzein intermediates in a process involving a racemase plus daidzein reductase, dihydrodaidzein reductase and tetrahydrodaidzein reductase (Tsujii et al. 2012; Schröder et al. 2013). Indeed, equivalent and very similar racemase and reductase enzymes have been found in every equol-producing strain characterized so far, always encoded by the corresponding genes organized in a 10 kb operon-like structure (Shimada et al. 2011; Schröder et al. 2013; Flórez et al. 2019).

Equol-producing bacteria are fastidious and extremely oxygen-susceptible, which hinders their use in the biotechnological production of equol at the industrial scale (Clavel, Lepage and Charrier 2014). Cloning the genetic machinery of equol producers into model organisms might, however, overcome this problem. The genes of a Lactococcus garvieae equol-producing strain coding for the above three reductases (dzz, ddr and tdr) have already been cloned and expressed in Escherichia coli (Shimada et al. 2010, 2011, 2012), as have those of S. isoflavoniconvertens and Egerthella sp. YY7918, with all the resulting recombinant bacteria producing this equol (Schröder et al. 2013; Kawada et al. 2016; Lee et al. 2017; Peiróteín, Gaya and Landete 2020). Large-scale production would allow for a greater number of human trials to evaluate the health benefits of equol, and help extend them to individuals beyond those that harbor equol-producing microbes in their gut. In particular, lactic acid bacteria (LAB) species and strains have qualified presumption of safety (QPS) status (EFSA BIOHAZ Panel 2020). They, therefore, offer a potentially safe vehicle for producing equol. Genetically modified LAB capable of developing in soybean extracts (Delgado et al. 2019) might even be used to produce fermented soybean products enriched in equol.

The present work reports the cloning in suitable vectors of a synthetic DNA fragment based on sequences of the equol operon from A. equolifaciens DSM19450T and its subsequent expression in E. coli, Lactcaseiibacillus casei and Lactococcus lactis (Maruo et al. 2008). This fragment contains the genes dzz, ddr and tdr under their native expression signals, preceded by the racemase gene under the control of a strong constitutive promoter from L. lactis. The conversion of daidzein into equol was seen in the recombinant E. coli when dihydrodaidzein was used as a substrate. No equol was ever produced by recombinant cells of L. lactis.

**MATERIALS AND METHODS**

**Plasmids, bacteria and culture conditions**

The bacterial strains and plasmid vectors used in the present work are summarized in Table 1. Escherichia coli DH10B was grown in Luria Bertani (LB) or 2xTY broth media with shaking at 37°C. Lactococcus lactis NZ9000 and Lactcaseiibacillus casei BL23 were grown statically at 32°C in M17 medium (Biokar, Beauvais, France) supplemented with 1% (w/v) glucose (GM17) and MRS (Merck, Darmstadt, Germany), respectively. Agar (2% w/v) was added to the media when required. Media were also supplemented with antibiotics for the selection of transformants and plasmid maintenance (100 μg/mL ampicillin and 300 μg/mL erythromycin for E. coli, and 2.5 or 5 μg/mL of erythromycin, respectively, for L. lactis and L. casei).

**Design of synthetic DNA**

The sequence of the equol biosynthesis gene cluster from Adlercreuzia equolifaciens DSM19450T was retrieved from GenBank (accession number NC_022567.1). The sequence of four open reading frames (ORFs) thought to be involved in the synthesis of equol by their homology to equol sequences from other species, including the racemase gene (AEOU_2234), and those encoding the downstream reductases tetrahydrodaidzein reductase (drr, AEOU_2231), dihydrodaidzein reductase (ddr, AEOU_2230) and daidzein reductase (dzz, AEOU_2228; Fig. 1), were codon-optimized with the NG Codon System (Synbio Technologies; Monmouth Junction, NJ) using default settings for E. coli. The native intergenic expression signals were left in place, but the first gene (the racemase) was located under the control of the constitutive promoter P59 from L. lactis (van der Vossen, van der Lelie and Venema 1987). To facilitate cloning, some restriction enzyme sites were removed from or added to the sequence, and 20 bp flanking sequences identical to those flanking the multiple cloning site of pUC57 were annexed. A final synthetic DNA consisting of 5206 bp (Figure S1, Supporting Information) was synthesized at Synbio Technologies (Monmouth Junction, NJ).

**DNA manipulation and cloning**

General procedures for in vitro DNA manipulation were followed essentially as described by Sambrook and Russell (2001). Restriction endonucleases (Takara, Otsu, Shiga, Japan) and T4 DNA ligase (Invitrogen, Carlsbad, CA) were used as recommended by their manufacturers. DNA from agarose gels was purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare Biosciences, Buckinghamshire, UK). The In-Fusion cloning kit (Clontech, Mountain View, CA) was used according to the manufacturer’s instructions to join the synthetic DNA and the linearized pUC57.

Electrocompetent cells of E. coli DH10B were prepared as reported by Sambrook and Russell (2001). Electrocompetent L. casei and L. lactis cells were prepared according to the procedure by Holo and Nes (1989). Electrotransformation (electroporation) was performed using a Gene Pulser apparatus (Bio-Rad, Richmond, CA) following standard protocols for Gram-negative and Gram-positive bacteria. White/blue screening for pUC57 in E. coli was performed on LB plates supplemented with appropriate antibiotics, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 20 mg/mL; Sigma-Aldrich, St. Louis, CA) and isopropyl-ß-D-thiogalactopyranoside (0.5 M; Sigma-Aldrich). Transformants of L. lactis and L. casei were selected on GM17 or MRS agar plates with erythromycin.

Plasmid DNA from E. coli was isolated and purified as described by Sambrook and Russell (2001). Plasmids from L. casei and L. lactis were isolated using a modified version of the procedure described by O’Sullivan and Klænhammer (1993), involving the addition of 4 μL of mutanolysin (5 U μL) and 20 μL...
| Strain, synthetic DNA, plasmid | Relevant genotype, description or properties | Reference or source |
|--------------------------------|---------------------------------------------|---------------------|
| **Strains**                   |                                             |                     |
| Escherichia coli DH10B        | F<sup>−</sup>, mcrA, Δ(mrr-hsdRMS-mcrBC), ϕ80lacZΔM15, ΔlacX74, recA1, endA1, araΔ139, Δ(ara-leu)7697, galU, galK, λ<sup>+</sup>, rpsL(StrR), nupG | ThermoFisher Scientific |
| Lactococcus lactis NZ9000     | L. lactis subsp. cremoris MG1363 derivative pepN::nisRK; plasmid-free | Kuipers et al. (1998) |
| Lacticaseibacillus casei BL23 | Plasmid free strain                         | Acedo-Félix and Pérez-Martínez (2003) |
| E. coli pUC57                 | E. coli DH10B carrying pUC57; Am<sup>+</sup> | This study          |
| L. lactis pIL252              | L. lactis NZ9000 carrying pIL252; Em<sup>+</sup> | This study          |
| E. coli DH108-pUC57-equol     | E. coli DH108 carrying pUC57-equol; Am<sup>+</sup> | This study          |
| E. coli DH108-pIL252-pUC57-equol | E. coli DH108 carrying pIL252-pUC57-equol; Am<sup>+</sup> | This study          |
| L. lactis NZ9000-pIL252-pUC57-equol | L. lactis NZ9000 carrying pIL252-pUC57-equol; Em<sup>+</sup> | This study          |
| L. casei BL23-pIL252-pUC57-equol | L. casei BL23 carrying pIL252-pUC57-equol; Em<sup>+</sup> | This study          |
| **Plasmids**                  |                                             |                     |
| pUC57                         | pUC19-derived general cloning vector; Am<sup>+</sup> | ThermoFisher Scientific |
| pUC57-equol                   | Recombinant plasmid containing the synthetic DNA carrying the equol genes racemase, tdr, ddr and dzr preceded by P59 promoter cloned in pUC57; Am<sup>+</sup> | This study          |
| pIL252                        | Low copy-number cloning vector for Gram-positives, based on the replicon of pAM<sub>β</sub>1 from Enterococcus faecalis; Em<sup>+</sup> | Simon and Chopin (1988) |
| pIL252-pUC57-equol            | Recombinant plasmid containing pUC57-equol cloned in pIL252; Am<sup>+</sup>, Em<sup>+</sup> | This study          |
| **Synthetic DNA**             |                                             |                     |
| -equol                        | A DNA segment of 5206 nucleotides long (Figure S1, Supporting Information), including four ORFs based on the genome sequence of Adlercreutzia equolifaciens DSM19450<sup>T</sup>: racemase (AEQU<sub>2234</sub>), tetrahydrodaidzein reductase (tdr, AEQU<sub>2231</sub>), dihydrodaidzein reductase (ddr, AEQU<sub>2230</sub>) and daidzein reductase (drr, AEQU<sub>2228</sub>). | This study          |

Am<sup>+</sup>, resistance to ampicillin; Em<sup>+</sup>, resistance to erythromycin.

<sup>a</sup>Numbering of ORFs as in the A. equolifaciens DSM19450<sup>T</sup> genome accession number NC_022567.1.

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**Figure 1.** Substrates and enzymes of the equol biosynthesis pathway from daidzein. In color, enzymes (purple) involved in equol production and metabolites analysed (blue).
of proteinase K (20 mg/mL) to the lysis buffer before phenol-chloroform extraction. Constructs were verified by digestion with restriction enzymes, sequencing and sequence analysis.

Identification and quantification of isoflavones

Single colonies of each clone were selected and grown in 5 mL of appropriate liquid medium and incubated overnight under the species-specific conditions stated above. These cultures were then used for inoculating into 1% fresh medium supplemented with 200 μM of daidzein or dihydrodaidzein (both from LC Laboratories, Woburn, MA). As negative controls, plasmid-free bacterial hosts and strains carrying empty pUC57 and pLL252 plasmids were cultured under the same conditions. Isoflavones and their metabolites were extracted from the supernatant of triplicate cultures (except for the controls, for which only a supernatant was analysed) after filtering through a 0.2 μm PTFE membrane (VWR, Radnor, PA) according to the procedure by Guadamuro et al. (2015). Metabolite separation and detection were achieved by high-performance liquid chromatography (HPLC). Metabolite quantification was determined against calibration curves prepared using commercial standards (all from LC Laboratories).

RESULTS

To express the A. equolifaciens-based genes in E. coli, the synthetic DNA was first cloned in vitro in pUC57 using the Infusion technique and the cloning mixture transformed into electrocomptent E. coli cells. Purified colonies of several transformants were selected at random and verified by restriction analysis. Further, one clone carrying an insert of the right size was verified by sequencing. Recombination between the identical 20 bp sequences at the two ends of the synthetic DNA and the linearized pUC57 extremities, produced a new molecule (pUC57-equol) in which the whole multi-cloning site of the vector was replaced by 5166 bp of the synthetic DNA (Fig. 2). The pUC57-equol plasmid was transformed again into E. coli. The novel clones carrying this construct were inoculated in a culture medium supplemented with 200 μM of daidzein or dihydrodaidzein, the substrate precursor and an intermediate of equol, respectively (Fig. 1), and incubated at 37 °C. Supernatants from the cultures were sampled at 0, 8 and 24 h of incubation and analysed for daidzein, dihydrodaidzein and equol. No differences in bacterial growth in the presence of daidzein or dihydrodaidzein were observed among controls and clones (data not shown). As expected, the supplemented daidzein or dihydrodaidzein were recovered untransformed from control cultures, while the different E. coli clones converted these substrates into equol to different extents (Table 2 and Figure S2, Supporting Information). Cultures of pUC57-equol converted around 90% of the daidzein into equol at 24 h. However, and surprisingly, equol production by E. coli via this construct was only partial when dihydrodaidzein was used as a substrate (=15%). Despite these variations, the results showed that the synthetic A. equolifaciens-based equol sequences were functional in E. coli and allowed for the synthesis of equol from both daidzein and dihydrodaidzein.

The pUC57-equol construct and pLL252 vector were independently digested with EcoRI, ligated with T4 ligase and the ligation mixture transformed into electrocompetent E. coli cells. Cloning of pUC57-equol in pLL252 resulted in the formation of pLL252-pUC57-equol (Fig. 2). This construct could have been created in either L. lactis or L. casei, but this procedure is straightforward in E. coli. After verification, E. coli transformants carrying the pLL252-pUC57-equol were cultured in the presence of daidzein and dihydrodaidzein and the supernatants analysed as before. At 24 h, equol was recovered from E. coli cultures, indicating again that the biochemical pathway for equol production supplied by pLL252-pUC57-equol was functional, although the amount of equol produced with this construct was much lower than with pUC57-equol (Table 2).

The plasmid pLL252-pUC57-equol was then electrotransformed into L. casei and L. lactis. Transformants from the two strains carrying the construct were cultured in MRS or GM17, respectively, with daidzein or dihydrodaidzein and the supernatants analysed as before. Equol was detected in supernatants at 24 h of incubation for L. casei clones carrying pLL252-pUC57-equol only when dihydrodaidzein was used as a substrate (Table 2). No L. lactis clone producing equol from either daidzein or dihydrodaidzein was ever detected. To check whether these results derived from the non-functionality of the genes or mutations, the pLL252-pUC57-equol construct from L. casei and L. lactis was purified and transformed back into E. coli. The new E. coli transformants produced equol in amounts equal to that initially measured (10–12 μM).

DISCUSSION

The evidence that isoflavone-rich diets help reduce the risk of different syndromes and chronic diseases obtained in many intervention studies and via meta-analyses is far from conclusive (Fang et al. 2016; Akhlaghi, Zare and Nouriipur 2017; Zhou and Yuan 2015; Liu et al. 2014; He and Cheng 2013; Harland and Haffner 2008; Bolaños, Del Castillo and Francia 2010; Wei et al. 2012). The discrepancies in the results have been attributed to a large part of the human population possessing a non-equol-producing phenotype, due to the absence of equol-producing microbes in their intestine (Daily et al. 2019). Since equol has the strongest hormonal activity and the highest antioxidant action of all isoflavone metabolites (Setchell and Cole 2006), variation in the results of an intervention might thus be expected depending on the human population sampled. To be more certain about its health benefits, large-scale biotechnological production is necessary; this would overcome equol shortages, enabling more trials to be conducted (Selvaraj et al. 2004). However, equol is produced from the plant isoflavone daidzein by strict anaerobes with many nutritional needs (Salam et al. 2020), hampering their use in the industrial-scale of equol production, as a result of the requirement of high investment inexpensive ingredients and equipment (Clavel, Lepage and Charrier 2014). The ‘aerobic domestication’ of equol-producing strains has been suggested as a means of overcoming these difficulties (Zhao et al. 2011), as has the cloning of the equol production machinery into easily cultivable heterologous hosts (Tsujii et al. 2012; Schröder et al. 2013).

In the present work, the four synthetic genes based on sequences from the A. equolifaciens equol operon, drove equol production in E. coli when either daidzein or dihydrodaidzein was present as a substrate. This is not surprising; the expression of equol genes from other equol-producing species in E. coli has been reported before (Kawada et al. 2016; Schröder et al. 2013; Shimada et al. 2010, 2011, 2012). In agreement with the present results, equol production by recombinant clones at the μmol level has also been reported in other works (Lee et al. 2016; Li et al. 2018). This is attributed to the poor solubility of isoflavones (and thus daidzein) in aqueous systems (del Rio et al. 2013), which restrains using larger amounts of daidzein. This problem has recently been overcome by adding hydrophilic polymers to the culture medium (Lee et al. 2018). Nonetheless, the daidzein to
Figure 2. Physical map of the plasmid constructs obtained in this work. The synthetic DNA cloned in pUC57 carries four equol-related genes from A. equolifaciens DSM19450T: the genes *racemase*, *tdr*, *ddr* and *dzr*, which encode a racemase, and the tetrahydrodaidzein, dihydrodaidzein and daidzein reductase, respectively. The genes are preceded by the P59 promoter from *L. lactis* subsp. *cremoris* Wg2. Color key: in red, antibiotic resistance genes (Amr, ampicillin resistance; Emr, erythromycin resistance); in light blue, the origin of replication; in orange, the gene encoding the β-galactosidase-complementing peptide, including the multiple cloning site (MCS) in brown; in green, genes involved in equol production; in purple, P59 promoter. Relevant restriction enzyme sites are also indicated. Molecules are not drawn to scale.
Table 2. Daidzein and its derived metabolites (in μM) from daidzein- or dihydrodaidzein-supplemented cultures of recombinant E. coli DH10B, L. casei BL23 and L. lactis NZ9000 cells harboring equol-associated genes from A. equolifaciens DSM19450T.

| Substrate/strain/construct | Daidzein Sampling at 0 h | Dihydrodaidzein Sampling at 0 h | Equol | Daidzein Sampling at 8 h | Dihydrodaidzein Sampling at 8 h | Equol | Daidzein Sampling at 24 h | Dihydrodaidzein Sampling at 24 h | Equol |
|----------------------------|--------------------------|---------------------------------|-------|--------------------------|---------------------------------|-------|--------------------------|---------------------------------|-------|
| Daidzein (200 μM)          |                          |                                 |       |                          |                                 |       |                          |                                 |       |
| E. coli pUC57-equol        | 34.1 ± 1.1               | –                               | –     | 8.2 ± 0.9                | 105.8 ± 2.3                     | 0.2 ± 0.1 | –                        | –                  | 179.6 ± 38.6 |
| E. coli pIL252-pUC57-equol | 24.9*                    | –                               | –     | 10.4 ± 1.0               | 114.2 ± 14.2                    | 2.5 ± 0.4 | 9.4 ± 2.6                | 132.1 ± 11.3                  | 11.1 ± 2.1 |
| L. casei pIL252-pUC57-equol| 70.9 ± 1.7               | –                               | –     | 30.9 ± 5.1               | –                               | –     | 38.7 ± 9.6               | –                  | –       |
| L. lactis pIL252-pUC57-equol| 23.6 ± 2.4               | –                               | –     | 23.4 ± 2.6               | –                               | –     | 29.0 ± 0.6               | –                  | –       |
| E. coli                    | 25.8*                    | –                               | –     | 9.3*                     | –                               | –     | 44.5 ± 6.9               | –                  | –       |
| E. coli pUC57              | nd                       | nd                              | nd    | nd                       | nd                              | nd    | 147.4*                  | –                  | –       |
| L. casei                   | 67.9*                    | –                               | –     | 23.5*                    | –                               | –     | 35.4*                   | –                  | –       |
| L. lactis                  | 30.7*                    | –                               | –     | 31.9*                    | –                               | –     | 26.8*                   | –                  | –       |
| L. lactis pIL252           | nd                       | nd                              | nd    | nd                       | nd                              | nd    | 255.7*                  | –                  | –       |
| Dihydrodaidzein (200 μM)   |                          |                                 |       |                          |                                 |       |                          |                                 |       |
| E. coli pUC57-equol        | –                        | 181.0 ± 2.4                     | –     | 12.9*                    | 146.2 ± 32.3                    | 0.6*  |                           | 84.1 ± 5.9                    | 34.9 ± 2.1 |
| E. coli pIL252-pUC57-equol | –                        | 98.8 ± 25.8                     | –     | 4.7 ± 0.2                | 155.4 ± 49.2                    | 2.3 ± 0.3 |                           | 149.4 ± 8.2                    | 12.0 ± 2.7 |
| L. casei pIL252-pUC57-equol| –                        | 136.5 ± 3.1                     | –     | –                       | 126.1 ± 6.4                     | –     | 48.6 ± 2.9               | 4.8 ± 2.2                     | –       |
| L. lactis pIL252-pUC57-equol| –                       | 233.7 ± 37.7                    | –     | –                       | 201.7 ± 12.6                    | –     | 182.5 ± 1.0             | –                  | –       |
| E. coli                    | –                        | 193.6*                          | –     | 2.7*                     | 187.2*                          | –     |                           | 119.9*                      | –       |
| E. coli pUC57              | nd                       | nd                              | nd    | nd                       | nd                              | nd    | –                       | 175.2*                      | –       |
| L. casei                   | –                        | 191.2*                          | –     | –                       | 190.4*                          | –     | –                       | 176.6*                      | –       |
| L. lactis                  | –                        | 188.6*                          | –     | –                       | 191.3*                          | –     | –                       | 204.2*                      | –       |
| L. lactis pIL252           | nd                       | nd                              | nd    | nd                       | nd                              | nd    | –                       | 148.6*                      | –       |

nd, not determined; -, not detected or below the limit of quantification; *data from a single experiment.
equol conversion ratio of E. coli clones carrying pUC57-equol at 24 h (≈90%), higher than that obtained with the original strain A. equolifaciens DSM19450\(^\text{T}\) (Flórez et al. 2019), is considered a promising result for industrial scale-up implementation.

The fact that the recombinant E. coli cultures carrying pUC57-equol produced more equol than those carrying pIL252-pUC57-equol might be explained by the larger size of the latter construct, which may lead to a reduced copy number. However, it cannot be ruled out that the larger construct is less stable. Indeed, the pUC57-equol was occasionally seen to be somewhat unstable, lacking some clones the ability to produce equol. This instability might also account for the large variation in equol production seen between different cultures of replicates of the same clone, giving rise to high standard deviations. Certainly, instability has also been reported for equol genes from S. isoflavoniconverters in E. coli. As such, Lee and co-workers have shown that mutations in the ddr gene (encoding the dihydrodaidzein reductase) helped to stabilize the cloned genes, leading to greater equol production (Lee et al. 2016). Mutations in E. coli housekeeping genes have also been shown to promote equol formation in cultures. In that sense, a mutation in ydS, which codes for a putative electron-transfer flavoprotein-quinone oxidoreductase, has been shown to increase equol production by overcoming the latter compound’s inhibition of E. coli growth (Li et al. 2018).

Given that the equol operon of L. garvieae seems to have been acquired by horizontal transfer from an Eggerthellaceae species (Shimada et al. 2010, 2011), the expression of equol genes in other LAB species ought to be feasible. However, neither the present recombinant L. casei nor L. lactis strains produced equol from daidzein; indeed, daidzein was recovered (largely) untransformed from the LAB cultures at all sampling points. The fact that a small amount of equol was formed by L. casei from dihydrodaidzein, however, indicates that the dihydrodaidzein reductase and the tetrahydrodaidzein reductase enzymes were active, implying that the genes were correctly transcribed and translated. In contrast, no equol was ever detected in L. lactis cultures, irrespective of the substrate provided. The P59 promoter of L. lactis drives the expression of homologous and heterologous proteins in strains of E. coli (van der Vossen, van der Lelie and Venema 1987), L. lactis (Que et al. 2000; Quiñot-Martínez et al. 2010) and L. casei (Gold et al. 1996), suggesting the trouble does not lie at this point. Perhaps, then, some of the intergenic regions containing native A. equolifaciens expression signals were not properly identified by the LAB transcription or translation machinery. Moreover, the optimization of codon usage for E. coli could have caused the poor (or lack of) expression of some genes in LAB (the GC content of L. lactis [34%] is rather low compared to E. coli and L. casei [=50%]). Despite similarity in GC content, gene expression in L. casei, as compared to E. coli, might require specific signals (Bintsis 2018). Alternatively, gene-disrupting or inactivating mutations may have appeared. However, the fact that the transformation of the recombinant DNA from L. casei and L. lactis back into E. coli enabled the latter host to produce equol, indicates the constructs were still functional. Finally, all three reductases are thought to be intracellular enzymes, and transport systems for isoflavone and equol have yet to be identified (Maruo et al. 2008; Shimada et al. 2011; Schröder et al. 2013). As daidzein and its derivatives were analysed in culture supernatants, differences between E. coli and LAB species in the import of substrates and/or the secretion of the metabolic end products could also account for the divergences.

In conclusion, synthetic genes coding for equol production, based on sequences from A. equolifaciens, were cloned and successfully expressed in E. coli and LAB species. To our knowledge, this is the first time that equol genes from A. equolifaciens have been cloned and expressed in heterologous hosts. The recombinant E. coli clones produced equol from daidzein, while those of LAB strains did not, although recombinant L. casei did produce small amounts from dihydrodaidzein. Escherichia coli clones harboring pUC57-equol could already be used for the large-scale biotechnological production of equol. Attempts are currently being made to express new synthetic genes in LAB based on those of A. equolifaciens but with LAB-specific transcription and translation signals.

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SUPPLEMENTARY DATA
Supplementary data are available at FEMSLE online.

Conflicts of Interest. None declared.

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