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ARTICLE

Deglycosylation of flavonoid *O*-glucosides by human intestinal bacteria *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4

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Abstract Daidzin, daidzein 7-*O*-glucoside, is a major isoflavone in soybean and acts as a phytoestrogen. By intestinal bacteria dietary, daidzin is hydrolyzed to the aglycone daidzein and further converted to the more reduced metabolites, such as dihydrodaidzein, tetrahydrodaidzein, and equol. Human intestinal bacteria *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4, which convert daidzin to daidzein, were isolated under anaerobic condition, and identified by 16S rRNA gene sequence analysis. Changes of OD_{600} and pH were measured during the anaerobic growth, and deglycosylation kinetics of daidzin and genistin were measured by HPLC. Both bacteria also converted other isoflavone 7-*O*-glucosides, glycitin, ononin, and sissotrin, to their aglycones, glycitein, formononetin, and biochanin A, respectively. Apigetrin was deglycosylated to apigenin by these bacteria too. However, rutin, hesperidin, and naringin were not converted to the aglycones. Phylogeny analysis of the isolated strains also found that bacterial species identified by 16S rRNA gene sequence was not correlated with its metabolic ability of flavonoid biotransformation.

Keywords Daidzin · Deglycosylation · Genistin · Human intestinal bacteria · Flavonoid *O*-glycosides

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Introduction

Major dietary sources of flavonoids, polyphenolic compounds in plant, are fruits, vegetables, and beverages. Daily flavonoid intake is estimated at between a few milligrams and grams depending on socio-geographic distributions (Andersen and Markham 2006). The growing interests in the flavonoid research are mainly due to the beneficial effects of maintaining health and preventing disease. For example, a plethora of studies demonstrated potent biological activities of flavonoids including anti-oxidant, anti-cancer, anti-inflammation, anti-atherogenic, and estrogenic effects (Chiang and Pan 2013; Kim and Han 2013; Jakhar et al. 2014; Kim et al. 2015). However, flavonoids are predominantly found as *O*- or *C*-glycoside forms and dietary flavonoid glycosides are to be metabolized to their aglycones mainly by human intestinal bacteria before absorption to the body. The aglycones are more rapidly absorbed than the glycosides (Izumi et al. 2000), and generally have better biological activities than the glycosides (Kim et al. 1988).

Daidzin is a major isoflavone *O*-glycoside in soybean and can be further metabolized to (*S*)-equol in human intestine (Wang et al. 2005; Kim et al. 2009, 2010). (*S*)-Equol is known to be more estrogenic, anti-carcinogenic, and anti-oxidant than its metabolic precursors (Shutt and Cox 1972; Mitchell et al. 1998). The first step of daidzin biotransformation involves hydrolysis of *O*-glycosidic bond of daidzin, and the aglycone daidzein is produced by human intestinal bacteria. A series of further reductive biotransformation of daidzein by intestinal bacteria is known to produce (*S*)-equol and *O*-desmethylangolensin (Fig. 1). Hur et al. (2000) first reported two bacterial strains of *Escherichia coli* HGH21 and Gram-positive HGH6 that

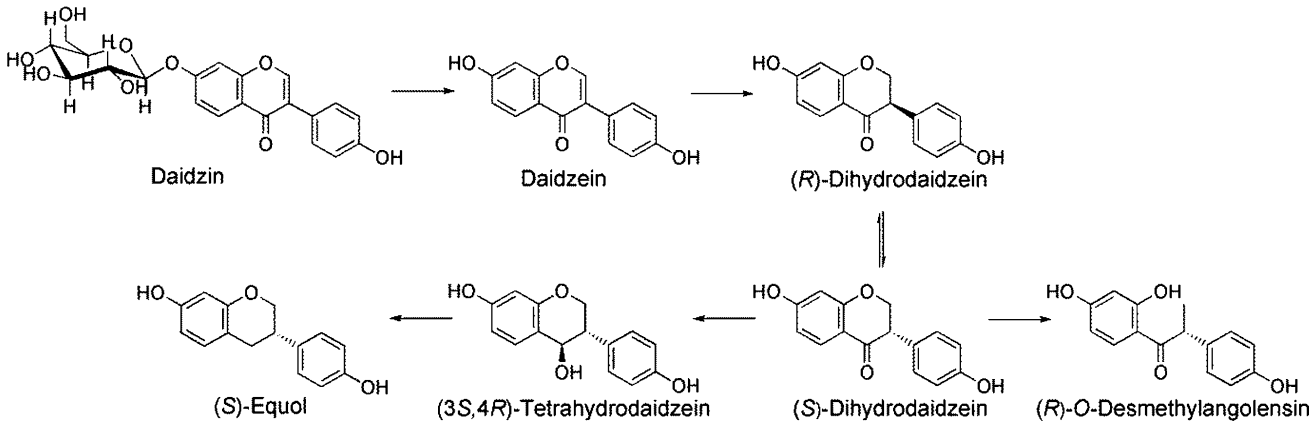


Fig. 1 Metabolic pathway of daidzin to (*S*)-equol by intestinal bacteria. After deglycosylation of the *O*-glycosidic bond, daidzein is stereospecifically reduced to (*R*)-dihydrodadizein (Park et al. 2011). Racemization between (*R*)-dihydrodaidzein and (*S*)-dihydrodaidzein provides substrate for the production of (*R*)-*O*-desmethylangolensin (Kim and Han 2014) and (3*S*,4*R*)-tetrahydrodaidzein (Shimada et al. 2010)

deglycosylated daidzin and genistin to the corresponding aglycones. Since then, only a few daidzin-deglycosylating bacteria, including *Clostridium*-like TM-40 (Tamura et al. 2007), *Coprobacillus* sp. MRG-1 (Park et al. 2011), *Lachnospiraceae* sp. CG19-1 (Braune and Blaut 2011), and *Eubacterium cellulosolvens* (Braune and Blaut 2012), were isolated from human fecal samples. Therefore, it is necessary to isolate and study more human intestinal bacteria involved in the biotransformation of daidzin and other flavonoid glycosides.

During the study of microbial biotransformation of flavonoids, two new daidzin-metabolizing bacteria, *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4 were isolated from human fecal samples. In this paper, basic microbiological characterizations of two isolates, biotransformation kinetics of daidzin and genistin, and substrate specificity of deglycosylation by these two bacteria were reported. In addition, phylogeny analysis of isoflavone-metabolizing bacteria using the available 16S rRNA gene sequences were carried out to understand whether there exists any relationship between bacterial species and flavonoid biotransformation activity.

Materials and methods

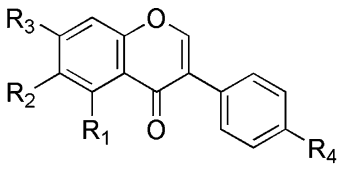
The experimental protocol was evaluated and approved by the Institutional Review Board of Chung-Ang University (Approval Number: 1041078-201502-BR-029-01).

Chemicals

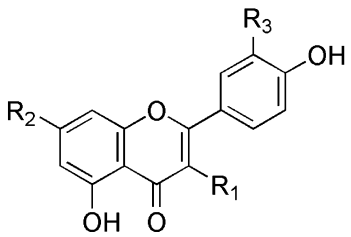
Flavonoids used for the study (Fig. 2), except rutin, were purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Rutin was purchased from Alfa Aesar (Lancashire, UK). Gifu anaerobic medium (GAM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan) and used for screening and growth media. GAM broth was prepared by manufacturer's instruction, and GAM plate was prepared with 15 % (w/w) of agar. Acetone, toluene, ethyl acetate, DMF, and acetic acid were purchased from Samchun pure chemical Co., LTD (Pyeongtaek-si, Gyeonggi-do, Korea). Methanol (HPLC grade) was purchased from Burdick & Jackson Laboratories, Inc. (Muskegon, MI, USA). Thin layer chromatography (TLC) silica gel 60 F_{254} plates were obtained from Merck (Merck, Darmstadt, Germany).

Isolation of daidzin-metabolizing bacteria

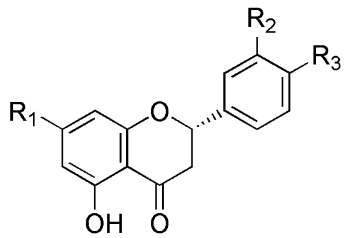
Fresh fecal samples from two healthy volunteers were collected in 4 mL of GAM broth media which were covered with sterilized mineral oil. The collected sample was immediately placed in anaerobic chamber and the isolation of daidzin-metabolizing bacteria was carried out according to the published method (Kim et al. 2015). For the activity check, the media were extracted with ethyl acetate and the dried residue was dissolved in 10 μL of methanol. Aliquot (2 μL) of methanol mixture was applied on the silica gel TLC plate with reference compound, daidzein. TLC plate was developed with a developing solution composed of toluene: acetone = 2:1. The metabolites on TLC plate were visualized with an UV transilluminator (254 nm). Fecal sample producing daidzein was diluted by several orders of magnitude, and the diluted samples were plated on GAM agar plates. After checking the activity of each diluted sample by TLC, single colonies were cultured in 200 μL of GAM broth media from the 10^{-8} plate. Activity



Genistin: R_1 = OH, R_2 = H, R_3 = O-Glu, R_4 = OH
Genistein: R_1 = OH, R_2 = H, R_3 = OH, R_4 = OH
Glycitin: R_1 = H, R_2 = OCH_3, R_3 = O-Glu, R_4 = OH
Glycitein: R_1 = H, R_2 = OCH_3, R_3 = OH, R_4 = OH
Ononin: R_1 = H, R_2 = H, R_3 = O-Glu, R_4 = OCH_3
Formononetin: R_1 = H, R_2 = H, R_3 = OH, R_4 = OCH_3
Sissotrin: R_1 = OH, R_2 = H, R_3 = O-Glu, R_4 = OCH_3
Biochanin A: R_1 = OH, R_2 = H, R_3 = OH, R_4 = OCH_3



Apigetrin: R_1 = H, R_2 = O-Glu, R_3 = H
Apigenin: R_1 = H, R_2 = OH, R_3 = H
Rutin: R_1 = O-Rut, R_2 = OH, R_3 = OH
Quercetin: R_1 = OH, R_2 = OH, R_3 = OH



Hesperidin: R_1 = O-Rut, R_2 = OH, R_3 = OCH_3
Hesperetin: R_1 = OH, R_2 = OH, R_3 = OCH_3
Naringin: R_1 = O-Rut, R_2 = H, R_3 = OH
Naringenin: R_1 = OH, R_2 = H, R_3 = OH

Fig. 2 Molecular structures of substrates and biotransformation metabolites cited in this study. *O-Glu O*-Glucose, *O-Rut O*-Rutinose

of each colony was checked as described above, and screening process was repeated until pure bacterium was obtained. The isolated strains, named as *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4, were deposited in the Korean Collection for Type Cultures (KCTC) under accession number KCTC15109 and KCTC15386, respectively.

16S rRNA gene sequencing and phylogeny analysis

Each bacterium was inoculated on the GAM agar plate in anaerobic chamber and grown for 1 day. DNA extraction, PCR, sequencing, and assembling for partial 16S rRNA sequence (1383 and 1352 bp) were performed by Macrogen Inc. (Seoul, Korea) Sequencing service. The 16S rDNA sequences were analyzed using NCBI's BLASTN tool with megablast program to identify the bacterial strain from the sequence similarity (Altschul et al. 1990). The sequence of 16S rDNA was deposited with the strain names of *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4 in GenBank database under accession number KF803556 and KF803557, respectively. The phylogenetic tree of daidzin-metabolizing intestinal bacteria was constructed by the neighbor-joining method using MEGA 6 program (Tamura et al. 2013). The evolutionary distances were computed using the maximum composite likelihood approach (Tamura et al. 2004). The other 16S rDNA sequences of *Enterococcus* and *Lactococcus* species involving daidzin metabolism were retrieved from NCBI website.

Growth curves and pH changes

Pure bacterial culture (OD_{600} = 1.2–1.4, 400 μL) of the subcultured isolates was inoculated into 20 mL of GAM broth media, and the media were incubated at 37 °C under anaerobic condition. In every 30 min, OD_{600} and pH were measured. UV–Vis spectrophotometer S-3100 (Scinco, Seoul, Korea) was used for the measurement of OD_{600}, and Laqua pH/ION/COND meter F-74BW (Horiba Scientific, Kyoto, Japan) was used for the measurement of pH.

Kinetics and substrate specificity of flavonoids deglycosylation

For the kinetics study of isoflavone biotransformation, daidzin and genistin, respectively, in DMF (10 mM, 15 μL) were reacted with 1.5 mL of bacterial culture (OD_{600} = 0.8). In every 10 min, reaction mixture (100 μL) was extracted with 1 mL of ethyl acetate. The ethyl acetate mixture was vortexed for 1 min and centrifuged for 5 min (10,770x*g*). After the supernatant (800 μL) was dried with vacuum concentrator (MICRO-CENVAC NB-503CIR; N-BIOTEK, Gyeonggi, Korea), the reaction product was dissolved in methanol (150 μL) and filtered through a 0.2-μm syringe filter (Grace, Columbia, MD, USA) for HPLC analysis.

To study the substrate specificity of deglycosylation by the isolated bacterial strains, each flavonoid *O*-glycoside (0.1 mM) was added to 100 μL of bacterial culture. After incubation in GAM media for 21 h, the reaction mixture was extracted with 1 mL of ethyl acetate. For the substrate that did not show the conversion, the reaction medium was incubated for 6 days. The analyte for HPLC analysis was prepared by ethyl acetate extraction as described above.

General HPLC methods

The reaction products were analyzed by Finnigan Surveyor Plus HPLC–DAD system (Thermo Scientific, Waltham, MA) equipped with a photodiode array detector (PDA Plus) and a C18 reversed-phase column (Hypersil GOLD 5 μm, 4.6 by 100 mm; Thermo Scientific, Waltham, MA). The mobile phase was composed of 0.1 % acetic acid in deionized water (solvent A) and 0.1 % acetic acid in methanol (solvent B). Following the injection of 10 μL of analyte, the elution profile started with an A:B ratio of 80:20 (v/v) for 1 min. The solvent gradient was then changed linearly to 50:50 (v/v) over 9 min, to 30:70 (v/v) over next 4 min, and hold at 30:70 (v/v) for 1 min. The eluent flow rate was 1.0 ml/min.

Results

Isolation and identification of human intestinal bacteria *Enterococcus* sp. MRG-2 and *Lactobacillus* sp. MRG-IF-4

Two bacteria metabolizing daidzin to daidzein were isolated from human fecal samples under anaerobic condition. When the bacterial shapes of two isolates were observed under a microscope, round and rod forms were identified (Supplementary Fig. S1). The 16S rRNA genes of the subcultured isolates were cloned and sequenced for the bacterial identification, and two different strains of MRG-2, and MRG-IF-4 were identified. The partial 16S rRNA gene sequence of MRG-2 (1383 bp) was identical to several *Enterococcus* spp. including *Enterococcus faecium* and *Enterococcus durans*. Therefore, MRG-2 was assigned as *Enterococcus* sp. MRG-2. The partial 16S rRNA gene sequence of MRG-IF-4 (1352 bp) was found identical to those of *Lactococcus lactis* bacteria. Therefore, MRG-IF-4 was assigned as *Lactococcus* sp. MRG-IF-4.

Because several isoflavone-metabolizing human intestinal bacteria isolated by us and others were identified as either *Enterococcus* sp. or *Lactococcus* sp., we built a phylogenetic tree to find the correlation between bacterial species and isoflavone-metabolizing activity. In Fig. 3, the bacterium with isoflavone reductase and flavonoid *O*-deglycosidase activity was labeled with an open square and open circle, respectively. Bacterium exhibiting both flavonoid *O*- and *C*-deglycosidase activities was labeled with a closed circle. Phylogenetic tree analysis of eight bacteria showed a good correlation between 16S rRNA gene sequences alignment and biotransformation activity of flavonoids. But, flavonoid biotransformation activity was not correlated with bacterial species as shown in Fig. 3. For example, the deposited partial 16S rRNA gene sequences of three *L. lactis* species, strain MRG-IF-4, MRG-IF-3, and MRG-IFC-1 (Kim et al. 2015), were compared. *Lactococcus* sp. MRG-IF-4 isolated in this study showed only flavonoid *O*-deglycosylation activity, but *Lactococcus* sp. MRG-IFC-1 with 100 % partial 16S rDNA sequence identities to the MRG-IF-4 exhibited *C*-deglycosylation activity as well. Furthermore, *Lactococcus* sp. MRG-IF-3 exhibited the same *O*-deglycosylation activity as the MRG-IF-4 strain showed only 96 % partial 16S rDNA sequence identities.

Growth curves of *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4

The growth curves of the isolated bacteria in GAM medium under anaerobic conditions at 37 °C were obtained by monitoring the absorption changes at 600 nm using a spectrophotometer. The cell growths of *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4 in GAM broth media were almost identical, except the OD_{600} values at the stationary phase. Cell densities of the former and the latter were found OD_{600} 3.3 and 2.7, respectively, at the stationary phase. Both strains reached the stationary phase in 4 h after 1 h of lag phase at 37 °C (Fig. 4). The pH values of the media with *Enterococcus* sp. MRG-2 was decreased from pH 6.9–5.8 during the exponential growth phase, and reached plateau of pH 6.0 at the beginning of stationary phase. In case of *Lactococcus* sp. MRG-IF-4, the pH value of growing media changed from pH 6.9–5.7, and to pH 5.9 during the exponential growth phase. At the beginning of stationary phase, it decreased to pH 5.7 again.

Kinetics and substrate specificity of flavonoids deglycosylation

Kinetics of daidzin and genistin biotransformation was studied by HPLC analysis. Deglycosylation of both daidzin and genistin by *Enterococcus* sp. MRG-2 was initiated after 60-min incubation with substrates. Genistin hydrolysis was a little faster than daidzin hydrolysis, and both reactions were completed within 3 h (Fig. 5A). The same deglycosylation by *Lactococcus* sp. MRG-IF-4 was initiated much faster than *Enterococcus* sp. MRG-2, and the products' formation was observed after 20 min incubation. In case of *Lactococcus* sp. MRG-IF-4, deglycosylation of daidzin was much faster than that of genistin that the conversion of daidzin to daidzein was completed in 60 min (Fig. 5B). It appeared two bacteria have different substrate preference.

Substrate specificity of the isolated bacteria was tested in GAM media with flavonoid glycosides (Fig. 2), to find out whether the deglycosylation activity by *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4 was applicable

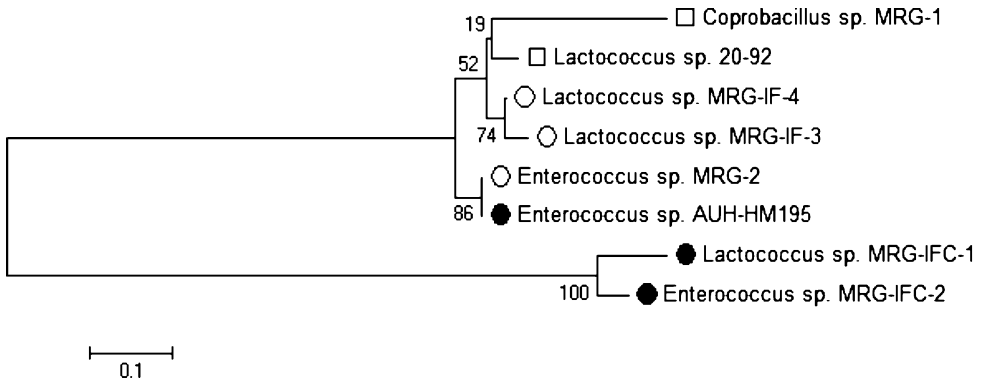


Fig. 3 Molecular phylogenetic tree analysis of isoflavone-metabolizing bacteria; *Coprobacillus* sp. MRG-1 (HQ687764), *Lactococcus* sp. 20–92 (AB300504), *Lactococcus* sp. MRG-IF-4 (KF803557), *Lactococcus* sp. MRG-IF-3 (KF803553), *Enterococcus* sp. MRG-2 (KF803556), *Enterococcus* sp. AUH-HM195 (EU919863), *Lactococcus* sp. MRG-IFC-1 (KF803554), and *Enterococcus* sp. MRG-IFC-2 (KF803555). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 1245 positions in the final dataset

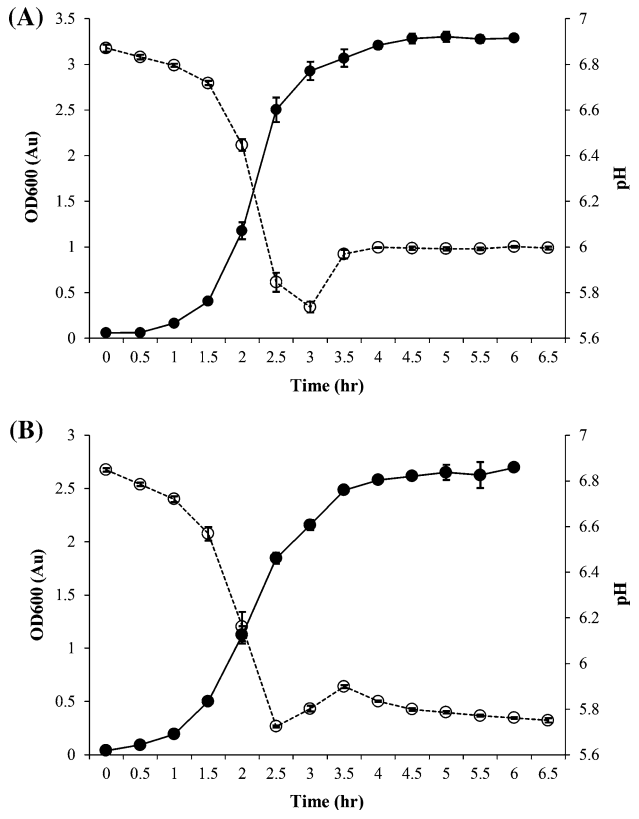


Fig. 4 Growth curves and pH changes of (**A**) *Enterococcus* sp. MRG-2 and (**B**) *Lactococcus* sp. MRG-IF-4

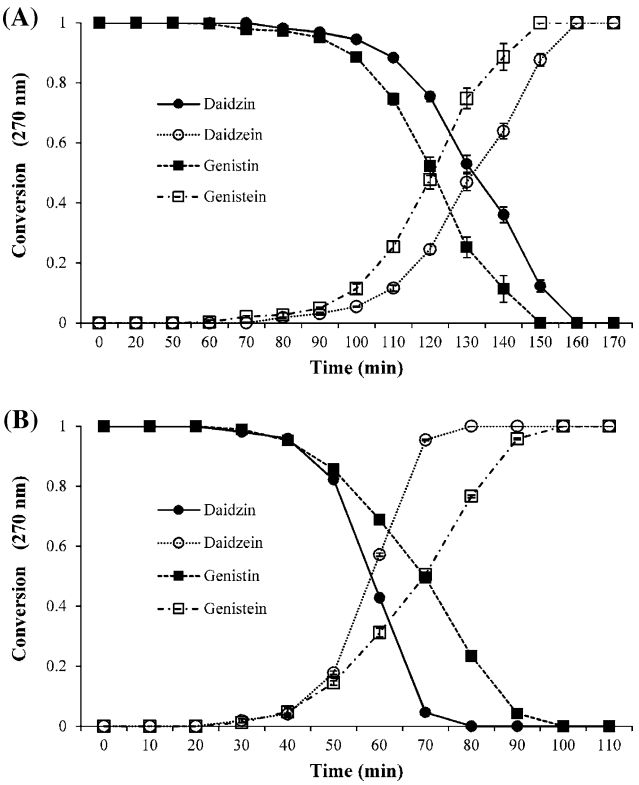


Fig. 5 Biotransformation of daidzin and genistin by (**A**) *Enterococcus* sp. MRG-2 and (**B**) *Lactococcus* sp. MRG-IF-4

to other flavonoid *O*-glycosides. The reaction products were identified by comparing the retention time and UV spectrum of each standard compound from the HPLC–DAD analysis (Supplementary Table S1). Both bacterial strains did not show any difference in the substrate specificity of the tested substrates. Isoflavone 7-*O*-glucosides, including glycitin, ononin, and sissotrin, were converted to their aglycones, glycitein, formononetin, and biochanin A, respectively. Apigetrin, flavone 7-*O*-glucoside, was also deglycosylated to apigenin. However, rutin, flavonoid 3-*O*-rutinoside, as well as hesperidin and naringin, flavanone 7-*O*-rutinosides, were not metabolized by both bacteria even after 6 day incubation (Supplementary Fig. S2).

Discussion

Since the discovery of (*S*)-equol, nonuniformity of bacterial metabolism in human intestine was soon realized and many studies emphasized the importance of human microbiota in the etiology of various disease, obesity, immune response, and other human health-related metabolisms (Nicholson et al. 2012). Besides, as bioinformatics approaches using high-throughput sequencing contribute significantly to the areas of food and clinical microbiology, identification of bioactive metabolites produced by specific intestinal bacteria has become important and prerequisite to investigate the microbiota-host interactions at the molecular level (Jacobs et al. 2009; Kim et al. 2014). For example, daidzein produced by deglycosylation of daidzin is metabolized to (*S*)-eqoul or *O*-desmethylangolensin via dihydrodaidzein intermediate by the specific intestinal bacteria (Fig. 1). The reported isoflavone metabolites were utilized for various metabolomic studies to investigate correlations among host physiology, host metabolism, and gut microbiota changes (Lee et al. 2012; Kobayashi et al. 2013). The genes involved in the biotransformation of daidzein and the metabolites were also cloned from the isolated intestinal bacteria for the biochemical research (Shimada et al. 2010; Tsuji et al. 2012; Schröder et al. 2013). Therefore, the isolation of new intestinal bacteria and characterization of the catalytic properties are of great significant.

In this study, human intestinal bacteria *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4 that convert daidzin to daidzein were isolated, and the flavonoid deglycosylation activity was studied. The phylogenetic analysis of these two and the other isoflavone-metabolizing bacteria found that bacterial strains identified by 16S rRNA gene sequences did not provide the conclusive evidence of the isoflavone-metabolizing ability. For example, different strains of *Lactococcus* species with high 16S rRNA gene homology were found to have different daidzin and daidzein biotransformation activities (Fig. 3).

During the characterization of the isolated bacteria, we have also found that both bacteria grow under aerobic conditions. Therefore, we carried out preliminary experiments related to the enzyme activity under aerobic conditions. First, the bacterial growths of *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4 were not discernible under the anaerobic and aerobic conditions. Secondly, deglycosylation activity of both bacteria was maintained in the presence of oxygen. Lastly, stronger deglycosylation activity was observed from the supernatants, when both cultured media were centrifuged down and daidzin deglycosylation activities of the supernatant and the cell lysate were compared by TLC analysis. Thus, it was preliminarily concluded that the enzymes responsible for the deglycosylation of flavonoids were not air-sensitive and secretory. Currently, biochemical study of the flavonoids deglycosylation enzymes produced by *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4 is underway.

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