RESEARCH LETTER

Isolation and identification of equol-producing bacterial strains from cultures of pig faeces

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Abstract

Transformation of daidzein to equol was compared during fermentation of three growth media inoculated with faeces from Erhualian piglets, but equol was produced from only one medium, M1. Two equol-producing strains (D1 and D2) were subsequently isolated using medium M1. Both strains were identified as *Eubacterium* sp., on the basis of morphological and physiological characteristics, and 16S rRNA gene sequence analysis showed that strains D1 and D2 were most closely related to previously characterized daidzein-metabolizing bacteria isolated from human faecal and rumen samples, respectively. This suggests that the ability to metabolize daidzein can be found among bacteria present within the mammalian intestine. The results provided the first account of conversion of daidzein directly to equol by bacterial species from farm animals. These strains may be of importance to the improvement of animal performance, and the use of medium M1 could provide a simple way to isolate bacterial strains capable of transforming daidzein into equol.

Introduction

Over the last two decades there has been increasing interest in isoflavonic compounds that are abundant in soy (Peters *et al.*, 1986; Phillips, 1992) because of their beneficial effects on human health. These compounds are structurally similar to the human female hormone, 17β-estradiol, and other steroid hormones, and can bind to oestrogen receptors in many tissues, acting as a weak agonist or antagonist (Kostelac *et al.*, 2003). It is widely believed that the relatively low incidence of breast and prostate cancers in China derives mainly from the high consumption of soy products. Oestrogenic activity can be enhanced after isoflavones are metabolized to more active compounds by the gut microbiota (Rowland *et al.*, 1999). The two major isoflavones found in soybeans are daidzin and genistin, which can be deglycosylated to daidzein and genistein, respectively, by human intestinal bacteria (Hur *et al.*, 2000). It has been proposed that daidzein is first transformed into dihydrodaidzein and then into *O*-desmethylangolensin (*O*-DMA) or equol (Chang & Nair, 1995; Heinonen *et al.*, 1999).

Equol, an intestinal metabolite of daidzein, has received particular attention, as it has greater phytoestrogenic activity than daidzein (Setchell *et al.*, 2002). It is produced exclusively by intestinal bacteria (Bowey *et al.*, 2003; Wang *et al.*, 2005a,b), but only approximately one-third of human individuals are estimated to harbour intestinal microorganisms capable of transforming daidzein into equol (Rowland *et al.*, 2000; Setchell *et al.*, 2002). The specific bacteria responsible for the production of equol are yet to be investigated, and few gut bacterial species have been identified that can metabolize daidzein. An anaerobic gram-positive strain, HGH6 (Hur *et al.*, 2000), and *Clostridium*-like bacterium TM-40 (Tamura *et al.*, 2007), which transform daidzein to dihydrodaidzein, and a *Clostridium* sp., strain HGH136 (Hur *et al.*, 2002), and *Eubacterium ramulus* (Schoefer *et al.*, 2002), which transform daidzein to *O*-DMA, have been isolated from human faeces. A human intestinal bacterium converting dihydrodaidzein into equol has also been isolated (Wang *et al.*, 2005a), and an anaerobic bacterium, Niu-O16, capable of converting daidzein to dihydrodaidzein, has been isolated from bovine rumen contents (Wang *et al.*, 2005b).

Although equol production has been established *in vitro* in human faecal samples (Rafii *et al.*, 2003; Atkinson *et al.*, 2004), information on equol-producing bacteria is limited. A bacterial strain, SNU-Julong 732, that converts dihydrodaidzein to equol was isolated from human faeces

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(Wang *et al.*, 2005a) and Decroos *et al.* (2005) obtained a mixed bacterial culture, also from human faeces, that could transform daidzein into equol. The microbial community was composed of four different taxonomic units, three of which could be cultured, identified as *Lactobacillus mucosae* EPI2, *Enterococcus faecium* EPI1 and *Finegoldia magna* EPI3. However, none of these species could produce equol from daidzein in pure culture, suggesting that uncultured or undetectable species were responsible for equol production. Recently, Minamida *et al.* (2006), for the first time, reported an anaerobic gram-positive strain capable of producing equol from daidzein. The strain, referred to as do03, was isolated from rat intestine and had 99% 16S rRNA gene sequence homology to the human intestinal bacterium SNU-Julong 732.

The fact that only limited bacterial strains capable of degrading daidzein and producing equol have been isolated and identified may be due to the need for anaerobic culture conditions and specific medium constituents. Indeed, most equol-producing mixed bacterial cultures or strains have been isolated from human faecal samples with BHI medium or GAM broth. Limited research also demonstrated that hydrogen gas, butyric acid, propionic acid and arginine could stimulate the conversion of daidzein to equol (Decroos *et al.*, 2005; Minamida *et al.*, 2006). However, few studies have investigated culture media suitable for the isolation of equol-producing bacteria.

It is widely established that intestinal microorganisms of humans and rats produce equol but, to our knowledge, no information is available on equol production by farm animals. This ability is of significance, as isoflavonic compounds and metabolites, such as daidzein and genistein, are beneficial to animal growth performance and health improvements (Han *et al.*, 2006). Daidzein could promote male animal growth, induce female mammary development and lactation, and improve the laying performance of laying birds. However, whether daidzein is further transformed to equol and the potential for equol in improving animal performance are so far unknown.

The aim of the present study was, therefore, to compare three media for equol production by microorganisms in pig faecal samples and to use this information to isolate and identify equol-producing bacteria from pig faeces. Successful isolation would facilitate the isolation of further strains of potential value in animal growth improvement.

Materials and methods

Equol production from pig faecal material

Growth media

Three media were investigated, referred to as M1, M2 and M3. M1 medium (Barcenilla *et al.*, 2000) contained (L^{-1} of distilled water) 10 g casitone, 2.5 g yeast extract, 4 g $NaHCO_3$, 2 g glucose, 1 g cysteine, 2 g cellobiose, 2 g soluble starch, 0.45 g K_2HPO_4, 0.45 g KH_2PO_4, 0.9 g $(NH_4)_2SO_4$, 0.9 g NaCl, 0.09 g $MgSO_4 \cdot 7H_2O$, 0.09 g $CaCl_2$ and 300 mL clarified rumen fluid. M2 is MRS medium (Klaenhammer & Kleeman, 1981), used to select lactic acid bacteria, and contained (L^{-1} of distilled water) 10 g peptone, 10 g beef extract, 5 g yeast extract, 20 g glucose, 2 g ammonium citrate tribasic, 5 g sodium acetate trihydrate, 5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g $MnSO_4 \cdot 4H_2O$ and 1 g of Tween-80. M3 was prepared according to Qian (1985) and contained (L^{-1} of distilled water) 10 g glucose, 10 g peptone, 5 g yeast extract, 3 g beef extract, 1 g $(NH_4)_2SO_4$, 0.2 g $MnSO_4$, 0.5 g $MgSO_4$, 1 g $CaCO_3$, 2 g NaCl, 1 g K_2HPO_4 and 1 g $NaHCO_3$. Anaerobic culture methods were those of Bryant (1972) using Hungate culture tubes, sealed with butyl rubber septa. Media were prepared and maintained anaerobically using O_2-free CO_2.

Experimental design

Preliminary tests were performed to determine pigs whose faecal material produced equol during *in vitro* incubation with daidzein as substrate (data not shown). Fresh faecal samples were collected from these pigs and placed immediately in warmed flasks that had been filled with carbon dioxide. The samples were processed within 1 h of collection, by adding sterile, anaerobic saline solution (1 : 4 faeces to saline), mixing in a blender for 60 s and filtering through a double layer of sterile cheesecloth. Five millilitres of the resulting mixture was then added to fermentation bottles containing 95 mL of M1, M2 or M3 medium with or without 200 $\mu g\,mL^{-1}$ daidzein. The dose of daidzein used was determined according to Zhao *et al.* (2003). Each faecal sample was inoculated into each medium in triplicate. For each medium, each faecal sample was further divided to inoculate treatment and control bottles. Three replicate bottles containing daidzein were inoculated with faecal material, serving as the treatment. Three replicate bottles containing no daidzein were also inoculated with pig faeces, to serve as control 1, to ensure that equol did not originate from the faeces. For each medium, another three replicate bottles containing medium and daidzein but no inoculum were also used, serving as control 2. All bottles were incubated at 37 °C for 48 h, after which the culture fluid was removed and stored at − 20 °C for HPLC analysis.

Determination of equol production

Equol concentration was determined according to Decroos *et al.* (2005). Culture fluid (1 mL) was extracted with diethylether and ether fractions in the supernatant were combined, evaporated to dryness, dissolved in 500 μL of

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50% (v/v) ethanol: dimethylsulphoxide and stored at 4 °C until analysis. Concentrations of daidzein and equol were determined using HPLC (Shimadzu Corporation, Tokyo, Japan). A 20 μL sample was injected and separated over an Agilent HC-C18 (Agilent Technologies). The temperature was set at 30 °C and the flow rate was maintained at 0.8 mL min^{-1}. Elution was isocratic with a mobile phase consisting of acetonitrile: methanol: water (30 : 20 : 50). Equol was detected at 205 nm and daidzein at 260 nm. UV-absorption spectra allowed identification of the peaks after comparison with pure standards. Calibration curves for quantification of daidzein and equol were constructed using pure standards obtained from Sigma (New York). The concentration of equol in the cultures was calculated according to a linear standard curve, which followed the equation $y = 371\,430x + 819\,544$ between the HPLC peak area (y) and the concentration of equol (x).

Isolation and identification of equol-producing bacteria

Bacteria were isolated using the roll-tube technique of Hungate (Hungate *et al.*, 1966), inoculating 0.05 mL culture suspension into roll-tubes containing 5 mL of medium M1 agar solidified with 0.09 g L^{-1} agar (Oxoid Ltd, Hampshire, UK). Tubes were rolled and then incubated at 37 °C for 48 h. Single colonies were transferred to anaerobic tubes containing 5 mL of M1 medium with 200 μg mL^{-1} daidzein, and samples were taken for HPLC analysis after incubation for 48 h at 37 °C. Bacterial strains that could metabolize daidzein to produce equol were further identified by morphological and physiological characterization, including carbohydrate fermentation patterns, according to Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974). The cellular morphology of the new isolates was observed by transmission electronic microscopy (TEM, Hitachi Corporation, Tokyo, Japan) using negative staining (Dai & Zhang, 1993).

DNA preparation and 16S rRNA gene sequencing

DNA was extracted from 48-h cultures of strains D1 and D2 following the method of Zhu *et al.* (2003) and Konstantinov *et al.* (2003). Almost full-length 16S rRNA genes were amplified from extracted DNA by PCR amplification using primers 8f (5′-CAC GGA TCC AGA GTT TGA T(C/T)(A/C)TGG CTC AG) and 1510r (5′-GTG AAG CTT ACG G(C/F)T ACC TTG CGA CTT) using the *Taq* DNA polymerase kit (Life Technologies) (Lane, 1991). Amplified products were purified with the QIAquick PCR purification kit and then sequenced commercially (Invitrogen, Shanghai, China). The assembled partial 16S rRNA gene sequences were compared with sequences from the GenBank database. A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the CLUSTAL X program and MEGA program. *Escherichia coli* was used as the outgroup.

Nucleotide sequence accession numbers

Bacterial 16S rRNA gene sequences for strains D1 and D2 were deposited in the GenBank database with accession numbers DQ904563 and DQ904564, respectively.

Results

Equol production during growth on three media

Faecal samples were inoculated into three different media and incubated anaerobically for 48 h at 37 °C. After incubation, culture material was extracted and subjected to HPLC analysis. The HPLC elution profiles provided a qualitative image of the daidzein metabolism by bacteria in the three media. Equol production was confirmed after comparing the retention time of the analysed compounds and commercially purchased equol. Equol was only detected after growth on medium M1 (Fig. 1), and no production was detected during the entire incubation period during growth on media M2 or M3. For all three media, no daidzein or equol was detected in control 1, which contained inocula and no daidzein, and daidzein but not equol was detected in control 2, that contained daidzein and no faecal inoculum.

Isolation and characterization of equol-producing bacteria

A total of 90 bacterial colonies were isolated from the Erhualian pig faecal culture. Each colony was inoculated into medium M1 with 200 μg mL^{-1} daidzein and incubated for 48 h under anoxic conditions at 37 °C. Cultures were harvested and equol was detected by HPLC. Two colonies (D1, D2) were found to transform daidzein into equol and, after subculturing, pure cultures were obtained, as determined by light microscopy. The HPLC profiles of daidzein and equol concentration during growth of strain D1 are shown in Fig. 2. The concentrations of equol produced by pure cultures of D1 and D2, and a mixed culture of faecal bacteria were 3.36 ± 0.48, 3.62 ± 0.29 and 25.2 ± 2.76 μg mL^{-1}, respectively.

According to Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974), phenotypic characteristics identified strains D1 and D2 as gram-positive, nonspore-forming, non-motile, strictly anaerobic, rod-shaped organisms (Fig. 3). Cells were 0.3–0.6 μm wide × 0.5–1.0 μm long and sometimes occurred in short chains of approximately two to four cells. Both strains formed circular, convex, smooth, white-opaque, 1–3-mm-diameter colonies on M1 agar. Both were capable of growth

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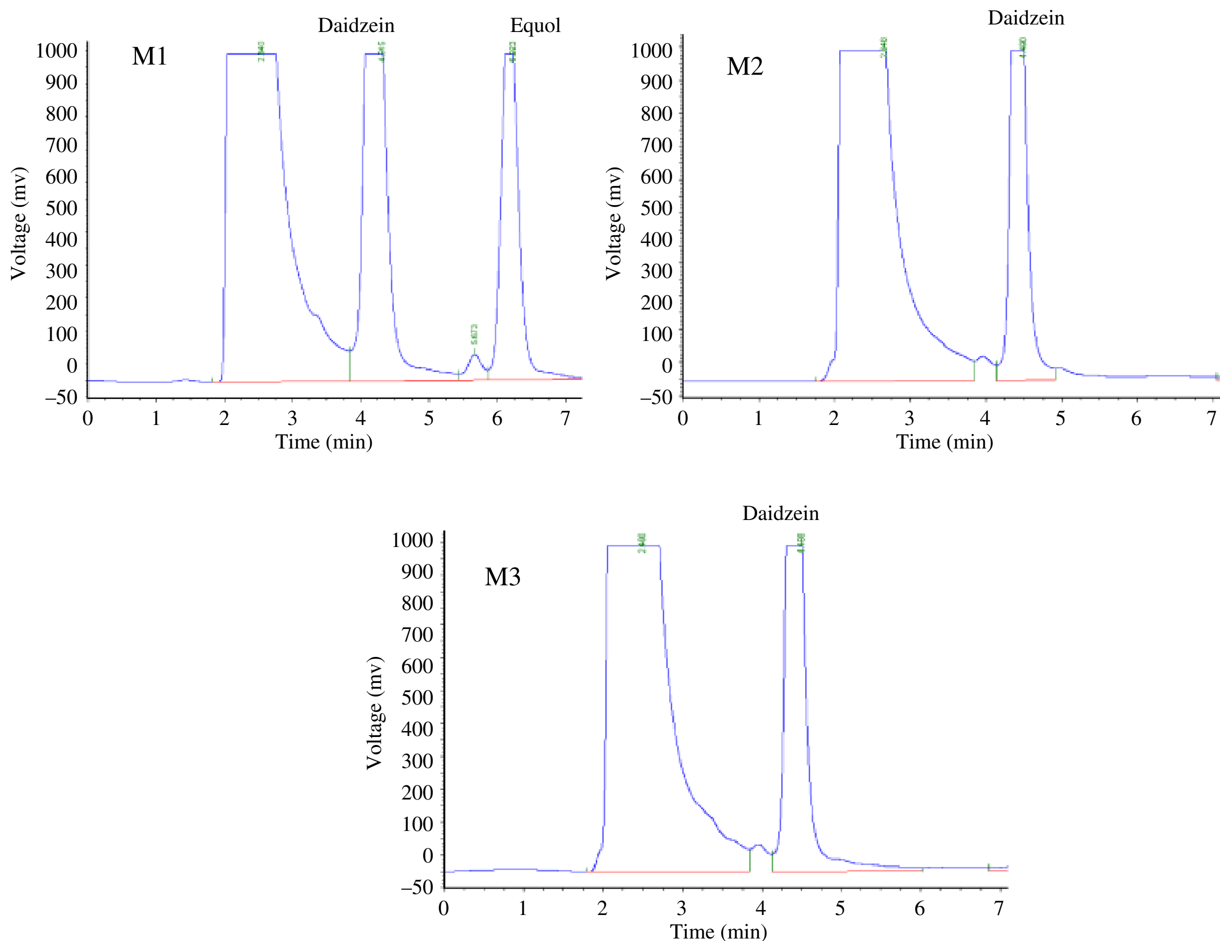

Fig. 1. HPLC analysis of microbial metabolism of daidzein in media M1, M2 and M3.

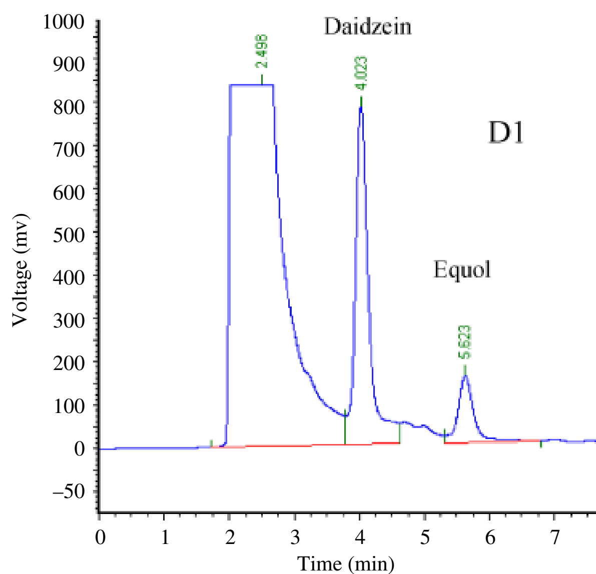

Fig. 2. HPLC elution profile of metabolism of daidzein and equol by strain D1 in M1 liquid medium under anaerobic conditions.

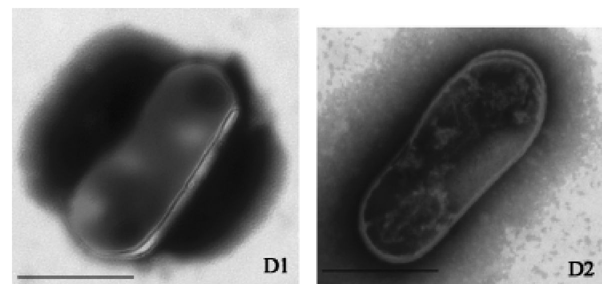

Fig. 3. TEM of strains of D1 and D2. Bar, 1μm.

at 45 °C, but not at 6.5% (w/v) NaCl or pH 9.6, and showed no evidence of indole production, nitrate reduction, gelatin liquefaction, catalase reduction or H_2S production. At the genus level, D1 and D2 were both identified as *Eubacterium* sp. Strain D1 fermented aesculin, maltose, salicin and starch, but could not ferment laetrile, arabinose, cellobiose, fructose, glucose, lactose, mannitol, mannitose, melezitose, raffinose, rhamnose, ribose, sorbierite, sucrose, and xylose. Strain D2 fermented maltose and starch, but could not

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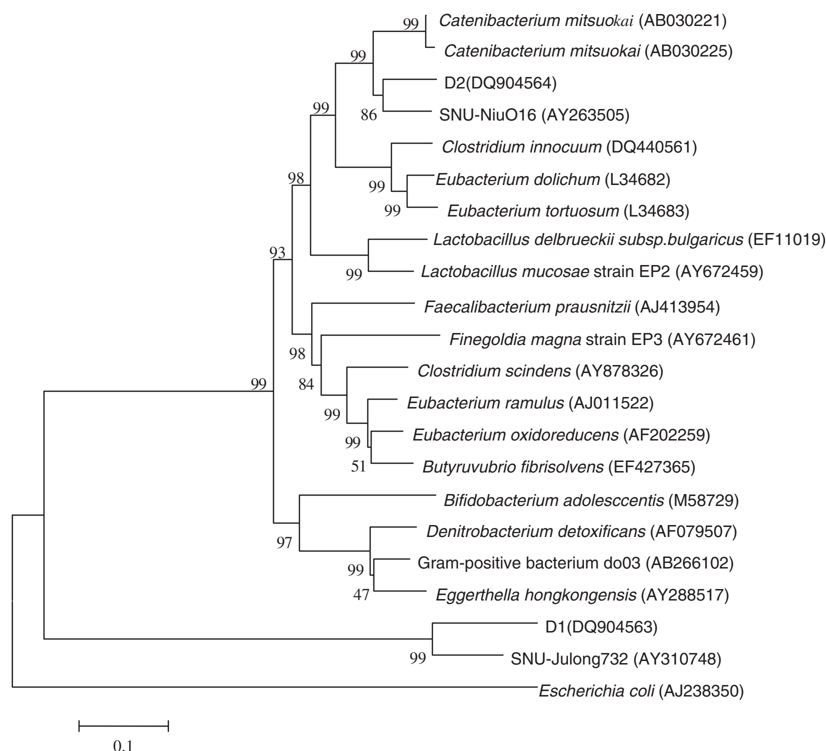

Fig. 4. Phylogenetic analysis of 16S rRNA gene sequences of strains D1 and D2. The scale bar represents genetic distance (10 substitutions per 100 nucleotides). The tree was constructed using the neighbour-joining analysis of a distance matrix obtained from a multiple-sequence alignment. Bootstrap values (expressed as percentages of the value for 100 replications) are shown at branch points; values of 97% or more were considered significant. *Escherichia coli* is used as the outgroup sequence.

ferment laetrile, arabinose, cellobiose, aesculin, fructose, glucose, lactose, mannitol, mannitose, melezitose, raffinose, rhamnose, ribose, salicin, sorbierite, sucrose, and xylose. Fermentation patterns did not permit species-level identification of strains D1 and D2.

Molecular characterization

Strains D1 and D2 were further characterized by analysis of almost full-length 16S rRNA gene sequences. The sequences of D1 and D2 were most closely related to uncultured bacterial species, with similarities of 99% for both. Phylogenetic analysis of these sequences placed strains D1 and D2 in the same clusters as strains SNU Julong732 and SNU NiuO16, respectively (Fig. 4).

Discussion

Intestinal bacterial metabolism of bioactive dietary compounds can alter intestinal activities (Atkinson *et al.*, 2005), which in turn can alter their potential to influence host health. *In vitro* studies suggest that equol is biologically more active than its precursor daidzein, and many investigators have focused on the oestrogenic potencies of equol, as reviewed by Setchell *et al.* (2002). Equol has a chiral carbon atom at position C-3 of the furan ring. It can occur as two distinct enantiomeric forms, *S*-equol and *R*-equol. Both enantiomers were bioavailable, but *S*-equol has a high affinity for oestrogen receptor β, whereas *R*-equol is relatively inactive (Setchell *et al.*, 2005). The present study using conventional HPLC was not able to seperate *S*-equol and *R*-equol. Setchell *et al.* (2005) showed that *S*-equol was the exclusive form of the equol product of human intestinal bacterial synthesis from soy isoflavones. However, whether pig faecel bacteria also exclusively produce *S*-equol requires further study. Chiral-phase HPLC chromatography would be an approach to further identify the equol form. Equol, usually referred to as *S*-equol, displays higher oestrogenic activity than daidzein and its antioxidant is greater than daidzein or genistein (Setchell *et al.*, 2002). The higher antioxidant activity of equol can lead to greater inhibition of lipid peroxidation and contributes to a reduction in the risk of cardiovascular disease (Peters *et al.*, 1986; Kostelac *et al.*, 2003). Research has demonstrated that equol is exclusively produced by intestinal bacteria (Bowey *et al.*, 2003; Wang *et al.*, 2005a, b). Thus, isolation and characterization of daidzein metabolizing and equol-producing microorganisms may lead to their use in production of higher levels of equol from daidzein.

A few equol-producing bacteria have been isolated. Strain SNU-Julong 732 isolated from human faeces can convert dihydrodaidzein to equol (Wang *et al.*, 2005a), and a mixed culture from human faeces (but not constituent single strains) can transform daidzein to equol (Decroos *et al.*, 2005). The only bacterial strain so far reported to convert daidzein directly to equol was recently isolated from rat faeces (Minamida *et al.*, 2006). The few equol-producing

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bacteria isolated have mostly been obtained from human faeces and rat intestinal samples. Our discovery of equol production and equol-producing bacteria from pig faeces is the first such report for farm animals. Daidzein has recently been introduced as a feed additive for farm animals in China. Many studies have demonstrated that daidzein could have anabolic effects on animal metabolism and performance, and could affect the neuroendocrine system and gut microbiota of animals, as reviewed by Han *et al.* (2006). In China, investigations showed positive effects of daidzein on animal growth performance. In studies on castrated male pigs, daidzein supplemented to diets at 5 mg kg^{-1} significantly increased weight gain by 59% and blood IGF-1 and testosterone levels were elevated by 51% and 18%, respectively (Guo *et al.*, 2002). This effect may be caused by the circulating isoflavonic metabolites following absorption from the intestine. It is possible that biological effects that have been associated with daidzein consumption in farm animals may be due to the biological properties of equol. Because intestinal bacteria are responsible for daidzein metabolism, equol production might be a marker of an intestinal bacterial profile associated with human health via unique metabolic activities of either the equol-producing bacteria, or other bacteria associated with their presence in the intestine (Atkinson *et al.*, 2005). Thus, although the purpose of this study was to isolate equol-producing bacteria, our study may also suggest that these daidzein-metabolizing bacteria may be involved in the beneficial effects of isoflavone or daidzein on farm animals. Further, manipulation of equol-producing bacteria and equol production in the intestine may have potential for improvement of farm animal performance. Conversion of isoflavones including daidzein occurs in the human colon, with consequences for human health (Cornwell *et al.*, 2004; Atkinson *et al.*, 2005). Conversion of these compounds in the porcine gut may result in increased concentrations of phytoestrogens in pork (Han *et al.*, 2006) and may also influence human health through the consumption of meat dishes. Indeed it is possible that the absorption of these products is more efficient when they become available directly from food sources in the upper gut than after release by microbial activity in the human colon.

It is widely accepted that daidzein is converted to dihydrodaidzein and/or equol by intestinal microorganisms. The two strains, D1 and D2, isolated from pig faeces in the present study and strain do03 isolated from rat faces by Minamida *et al.* (2006) are the only three bacterial strains in culture capable of transforming daidzein to equol. The few equol-producing isolates have been obtained using BHI medium (Hur *et al.*, 2000, 2002; Decroos *et al.*, 2005; Wang *et al.*, 2005a, b), ST medium (Schoefer *et al.*, 2002), EG medium (Tamura *et al.*, 2007) or GAM broth (Minamida *et al.*, 2006). In this study, three alternative media were assessed for their ability to select equol-producing bacteria. One of these media, M1, a relatively inexpensive medium previously used to isolate butyrate-producing bacteria (Barcenilla *et al.*, 2000), produced a stable mixed microbial culture that transformed daidzein to equol. Our approach therefore provides an alternate and inexpensive method for the isolation of these functional bacterial species.

Phenotypic analysis placed strains D1 and D2 within the genus *Eubacterium,* and 16S rRNA gene sequences showed close relationships with two isolates, SNU Julong732 and SNU NiuO16 with similar metabolisms, respectively. Both SNU Julong 732 and SNU NiuO16 have not been identified (Wang *et al.*, 2005a, b), but phylogenetic analysis revealed that strains D2 and SNU NiuO16 occupy the same cluster and are most closely related to the genus *Catenibacterium*. Strains D1 and SNU Julong732 occupy the same cluster, but are closely related to different unknown species. SNU Julong732, isolated from human faeces, could convert the isoflavone dihydrodaidzein to *S*-equol (Wang *et al.*, 2005a) and SNU NiuO16, isolated from rumen contents, could convert isoflavones daidzein and genistein to dihydrodaidzein and dihydrogenistein (Wang *et al.*, 2005b). The fact that strains D1 and D2 are most closely related to daidzein-metabolizing bacterial isolates from the human faeces and from the rumen, respectively, strongly suggests that this activity can be found among intestinal bacteria in mammalian animals.

The fact that equol production by D1 and D2 pure cultures was less than the mixed faecal bacteria may imply that other bacteria species in the mix culture may be involved in daidzein metabolism. Some may be involved in the first step of conversion, as SNU NiuO16 converted daidzein to dihydrodaidzein (Wang *et al.*, 2005b), or in the conversion of dihydrodaidzein to equol, as performed by strain SNU Julong732 (Wang *et al.*, 2005a). Decroos *et al.* (2005) demonstrated equol production by a mixed culture, but not by component pure cultures, and unknown bacteria species in mixed culture might complete conversion of daidzein to equol, thereby having an important role in equol production. Further, the medium conditions used in the experiments may not be optimal for other ‘fastidious’ bacterial groups that could also metabolize daidzein in the intestine, and are consequently not detected or not active *in vitro*. Moreover, different bacteria from different regions of the gut may take part in the bioconversion of daidzein into equol. On the other hand, daidzein may be transformed into equol through intermediate components or through different pathways involving bacteria associated with the tissue and not present in the faeces. Thus, the conversion of daidzein into equol could be completed collectively by different bacterial groups in the mammalian intestine. The present study provides the first account of the isolation of

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species capable of transforming daidzein into equol from farm animals. The mechanism about the bioconversion of daidzein into equol needs further investigation.

Phenotypic analysis suggests that the two strains isolated in the present study belonged to the genus *Eubacterium*. Many *Eubacterium* species are butyrate-producing bacteria. Our study also detected butyrate production of these two strains (data not shown) and we have previously shown that daidzein supplementation of feed can stimulate growth of some butyrate-producing-like bacterial species in the pig intestine (Yu *et al*., 2007). Together, this implies that some butyrate-producing bacteria in the intestine may harbour the ability to convert daidzein to equol. Thus, the present approach may increase the efficiency of isolation of equol-producing bacterial species.

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