Curr Microbiol (2012) 64:60–65
DOI 10.1007/s00284-011-0020-4

Comparison of the Effects of Antimicrobial Agents from Three Different Classes on Metabolism of Isoflavonoids by Colonic Microflora Using Etest Strips

John B. Sutherland · Brad M. Bridges · Thomas M. Heinze · Michael R. Adams · Patrick J. Delio · Charlotte Hotchkiss · Fatemeh Rafii

Received: 24 May 2011 / Accepted: 12 September 2011 / Published online: 18 October 2011

Abstract Daidzein (4′,7-dihydroxyisoflavone), a phytoestrogen found in soybeans mainly in the form of its glycoside daidzin, is metabolized by colonic bacteria to compounds with altered estrogenic activities, which may affect human health. Antibacterial agents used for the treatment of infections can alter the composition of bacterial populations in the colon and therefore can affect daidzein metabolism. To rapidly detect the effects of different concentrations of antibiotics on daidzein metabolism by colonic bacteria of monkeys and identify the subpopulation involved in daidzein metabolism, Etest strips containing antibacterial agents from three classes (tetracyclines, fluoroquinolones, and β-lactams) were used to eliminate the colonic bacteria that were susceptible to 0–32 μg/ml of each antibacterial agent and test the surviving bacteria for their ability to metabolize daidzein. The metabolism of daidzein by the colonic microflora was measured before and after the colonic bacterial population was exposed to antibacterial agents. The metabolites were detected by high performance liquid chromatography and mass spectrometry after incubation of the cultures for various times. Exposure of colonic microflora to antibiotics had various effects on daidzein metabolism. Tetracycline completely removed the bacteria metabolizing daidzein, metabolism of daidzein was not changed in cultures of bacteria after ceftriaxone treatment, and ciprofloxacin enriched for the bacteria metabolizing daidzein. In liquid cultures treated with various concentrations of ciprofloxacin, 4 μg/ml of ciprofloxacin favored the growth of bacteria that metabolized daidzein. This is the first time in which the Etest has been used to show that, whereas some antibiotics eliminate phytoestrogen-metabolizing bacteria in colonic microflora, others enrich them by eliminating the non-metabolizing strains in the population.

Introduction

The colonic microbiota contributes to many aspects of human health, including the prevention of colonization by pathogenic bacteria and the metabolism of various drugs and nutrients, including phytoestrogens [7, 11, 14, 17, 18, 20]. The isoflavone phytoestrogens, daidzin and genistin, the glycosylated forms of daidzein and genistein, are found in soybean. In the United States, in addition to soybean-based foods, other soy products with claims of cardiovascular and menopausal health benefits are marketed as nutritional supplements [3, 13].

Colonic bacteria from humans and other animals are able to metabolize isoflavones to compounds with different estrogenic activities [10, 21]. After daidzein is ingested, the colonic bacteria of monkeys and some humans may metabolize it to dihydrodaidzein and then to equol [9, 15]. Among the daidzein metabolites, equol has been suggested to have the most beneficial health effects [12, 13].

Electronic supplementary material The online version of this article (doi:10.1007/s00284-011-0020-4) contains supplementary material, which is available to authorized users.

J. B. Sutherland · B. M. Bridges · T. M. Heinze · F. Rafii (✉)
Division of Microbiology, National Center for Toxicological Research, Jefferson, AR, USA
e-mail: Fatemeh.Rafii@fda.hhs.gov

M. R. Adams
School of Medicine, Wake Forest University, Winston-Salem, NC, USA

P. J. Delio · C. Hotchkiss
Washington National Research Primate Center, University of Washington, Seattle, WA, USA

Antibiotics are often used for the treatment of bacterial infection, and exposure of colonic microbiota to antimicrobial agents may lead to elimination of highly sensitive strains, favoring the growth of microorganisms which are less sensitive to the drugs [17]. In human adults and children consuming soy products, different antibiotics affect the urinary concentrations of isoflavones differently, and the use of antibiotics in monkeys affects the metabolism of daidzein [1, 6].

The colonic microbial community, with 1800 estimated genera and around 15,000–36,000 bacterial species, is complex [5]. Detection of subpopulations in this complex that are involved in the metabolism of particular substrates is difficult. Also, finding the effects of different antibiotics on the metabolism of particular compounds involves growth of bacteria in the presence of many different concentrations of antibiotics in various tubes. Etest strips are used for the detection of levels of susceptibility of individual strains of bacteria to specific antibiotics. We have used Etest strips not only to rapidly find the subpopulation that is involved in the metabolism of daidzein in the colonic bacterial population, but also to detect the effects of antibiotic treatment on the metabolism of daidzein by the colonic microflora.

Materials and Methods

Chemicals and Sample Collection

Daidzein and equol (>98%) were obtained from Sigma Chemical Co. (St. Louis, MO). Dihydrodaidzein was synthesized according to a method described previously [9, 19].

Stool samples were taken from monkeys (*Macaca fascicularis*) and placed immediately in a tube containing 10% nonfat dry milk solution. The samples were overlaid with mineral oil to insure anaerobiosis, because the colonic bacteria metabolize daidzein under anaerobic conditions [16]. They were kept on dry ice during shipment and stored at −70°C before being used to inoculate Brain Heart Infusion (BHI) broth in the laboratory.

Effects of Antibiotics on Daidzein Metabolism In Vitro

To investigate the effects of antibiotics on the metabolism of daidzein, bacteria were spread on four BHI agar plates, using BHI broth that had just been inoculated with a frozen sample of the milk containing microbiota from a monkey. Etest strips (bioMérieux, Durham, NC), containing 0–32 μg gradients of ciprofloxacin, tetracycline, and ceftriaxone, were applied to three of the bacterial lawns. One other plate did not receive an Etest strip. The plates were incubated overnight under anaerobic conditions at 37°C.

Cells that grew inside the zone of inhibition around an Etest strip were considered resistant to that antibiotic. These resistant cells were scraped collectively from the plates and used to inoculate test tubes containing BHI broth with 10 μg/ml of daidzein, which had been dissolved in dimethyl sulfoxide. BHI broth containing daidzein was also inoculated with cells from the frozen sample of the original microbiota in milk. For controls, BHI broth, incubated with each bacterial mixture but without daidzein, and sterile BHI broth, with daidzein but without bacteria, were used. Samples were withdrawn from each of the inoculated and non-inoculated tubes at the time of inoculation; and the rest of the samples were incubated at 37°C in an anaerobic glove box (85% N_2, 10% CO_2, and 5% H_2) and sampled at intervals. The samples were extracted and the metabolism of daidzein by the colonic bacteria that had previously been grown in the presence of different concentrations of these antimicrobials was evaluated.

Extraction and Analysis of Samples by HPLC

The samples taken at the beginning of the experiment and at intervals were extracted three times with equal volumes of ethyl acetate. The extracts were dried over anhydrous Na_2SO_4 and the solvent was evaporated in vacuo. The residues were dissolved in 90% acetonitrile:10% water for analysis. HPLC analysis was performed with a Varian Star system (Varian, Inc., Palo Alto, CA). A Spherisorb C18 reversed-phase column (4.6 × 250 mm, S5, ODS-2, Phase Separation, Queensferry, Clwyd, Wales, UK) was used. The components of the mobile phase, (A) and (B), were 10 and 90% acetonitrile in water with 0.1% acetic acid, respectively. Following injection of the samples, the column was washed with 100% A for 10 min and then samples were eluted with a linear gradient of 10% to 90% B for 50 min at a flow rate of 1 ml/min [14]. The UV absorbance was monitored at 260 nm.

Liquid Chromatography/Mass Spectrometry (LC/MS) Analysis

Analyses were performed using a TSQ Quantum Ultra mass spectrometer (ThermoFinnigan, San Jose, CA) with an 1100 Series HPLC (Agilent Technologies, Palo Alto, CA) and a 5-μm Prodigy ODS-3 2.0 × 250 mm column (Phenomenex, Torrance, CA). The mass spectrometer was operated in negative-ion electrospray ionization (ESI) mode with the in-source collision-induced dissociation (CID) at 21 V to reduce solvent clustering. ESI conditions included spray voltage at 4.0 kV, capillary temperature 350°C, sheath gas pressure 20 psi, sweep gas 4 units, and auxiliary gas flow 40 units. Argon collision gas was set at 1.5 mTorr. Product-ion spectra were acquired in three scan

events: parent ion at *m/z* 253 with 25 eV collision energy for daidzein, parent ion at *m/z* 255 with 15 eV collision energy for dihydrodaidzein, and parent ion at *m/z* 241 with 15 eV collision energy for equol. Q3 was scanned from *m/z* 30 to 270 in 0.3 s for each scan event. The column heater was set at 30°C and the mobile phase, delivered at 0.2 ml/min, was a 40-min linear gradient from A (10% methanol:90% water) to B (95% methanol:5% water). Identification of the metabolites was confirmed by comparison of the product-ion spectra of metabolites with those of standards.

Results

Growth of Colonic Bacteria with Antibiotic Etest Strips

Application of Etest strips with tetracycline, ceftriaxone, and ciprofloxacin on the lawn of colonic bacteria inoculated on the surface of the plate under anaerobic conditions resulted in the elimination of bacteria in the population that were sensitive to 0–32 μg/ml of each of the antibacterial agents and the growth of bacteria that were resistant to antibiotics. The bacteria resistant to various concentrations of a drug grew as a ring in the zone surrounding each of the strips (Supplementary data, Fig. S-1) and were tested collectively for the ability to metabolize daidzein.

Effects of Different Antibiotics on Metabolism of Daidzein by Colonic Bacteria

HPLC elution profiles of the ethyl acetate extracts from cultures of the colonic microbiota of monkey, before and after exposure to each antibacterial agent, showed the metabolism of daidzein (Fig. 1). The major peaks found only in cultures containing daidzein were compared with standard compounds. The only two metabolites detected in cultures were equol and its precursor dihydrodaidzein. Daidzein eluted at approximately 34.8 min, dihydrodaidzein at 35.8 min, and equol at 36.8 min (Fig. 1). The UV absorption spectra (not shown) and product-ion spectra (Fig. 2) of the metabolites were identical to those of authentic standards of dihydrodaidzein and equol. HPLC analysis showed that daidzein was metabolized in 24 h in BHI broth cultures started directly from frozen samples of original flora in broth (Fig. 3). Mixed cultures from monkey fecal bacteria grown on the plates were also capable of metabolizing daidzein to equol after incubation. Various antibiotics affected the metabolism of daidzein by intestinal bacteria differently. When mixtures of colonies, taken from the zones of inhibition around Etest strips on BHI agar plates, were used to inoculate BHI broth containing daidzein, HPLC analysis showed differences in the rate of metabolism of daidzein by bacteria that had survived antibiotic treatment (Fig. 3). Tetracycline (TC) inhibited the daidzein-metabolizing bacteria, as was evident by the persistence of daidzein even after 96 h of incubation in cultures inoculated with bacteria that had survived exposure to tetracycline (Fig. 3). The amount of daidzein metabolized by bacteria that had survived treatment with ceftriaxone (TX) was similar to that metabolized by bacteria from untreated plates after 96 h (Fig. 3). Cultures that had survived treatment with ciprofloxacin (CI) metabolized daidzein more efficiently than untreated cultures (Fig. 3). These cultures, in addition to equol, also produced dihydrodaidzein, an intermediate in equol production that was not detected in cultures from the colonic bacteria of the same monkey without added ciprofloxacin (data not shown). The metabolism of daidzein was also measured in different concentrations of antimicrobial agents by inoculating cultures containing increasing concentrations of each of the antibiotics and measuring their effect on daidzein metabolism. Tetracycline inhibited daidzein metabolism; however, ciprofloxacin at 4 μg/ml (but not at 8 μg/ml) enhanced it (data not shown).

Numerous attempts were made to identify specific ciprofloxacin-resistant bacteria in the mixed culture that metabolized daidzein, by streaking daidzein-metabolizing mixtures on agar plates and examining individual colonies for daidzein metabolism. None of the isolated bacteria metabolized daidzein (data not shown).

Discussion

The colonic microflora is a complex ecosystem that metabolizes drugs and nutrients and modulates their effect on human health [4]. Daidzein, a phytoestrogen consumed because of its beneficial effect on human health, is metabolized by colonic bacteria to compounds with altered estrogenic activities [21]. The colonic bacteria come into contact with antibiotics used for treatment of bacterial infections and bacteria sensitive to them are eliminated during treatment, which could affect daidzein metabolism. Using a simple assay employing Etest strips for bacterial selection, we were able to find out the effects of treatment with different antibiotics on daidzein metabolism and also enrich for the population of bacteria that are involved in daidzein metabolism in the complex mixture. When the metabolism of daidzein by the intestinal microbiota was examined by HPLC and LC/MS, dihydrodaidzein and equol were the only major metabolites detected as the result of colonic bacterial activity. The minor peaks found by HPLC did not correspond to any known daidzein metabolites by either UV or LC/MS analysis.

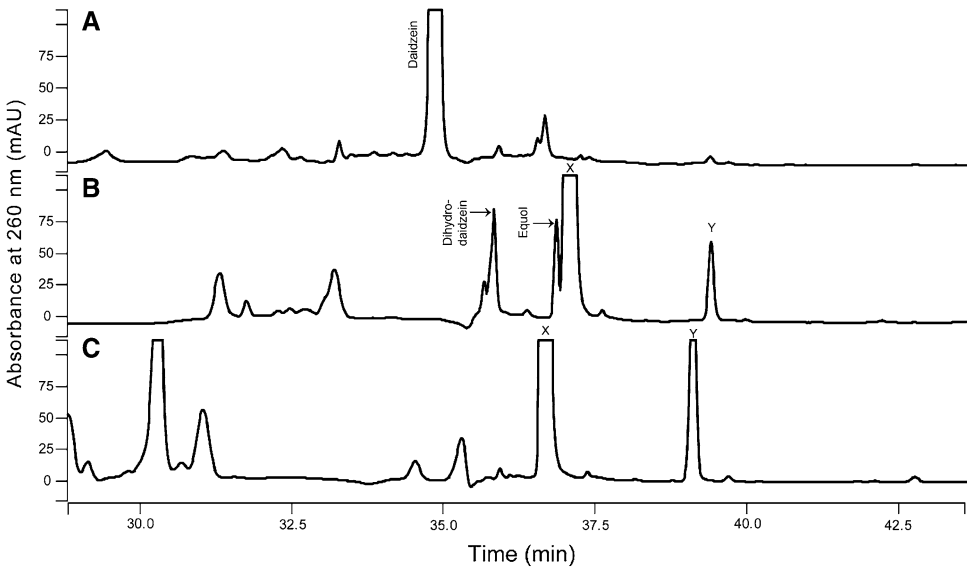

Fig. 1 HPLC elution profiles (UV = 260 nm) of ethyl acetate extracts from cultures of the colonic microbiota from a monkey, incubated under anaerobic conditions with or without 10 μg/ml of daidzein. (*A*) With daidzein at 24 h; (*B*) with daidzein at 48 h; and (*C*) without daidzein at 48 h. The unidentified peaks marked "X" and "Y", which were present in cultures with and without daidzein, are not related to daidzein metabolism

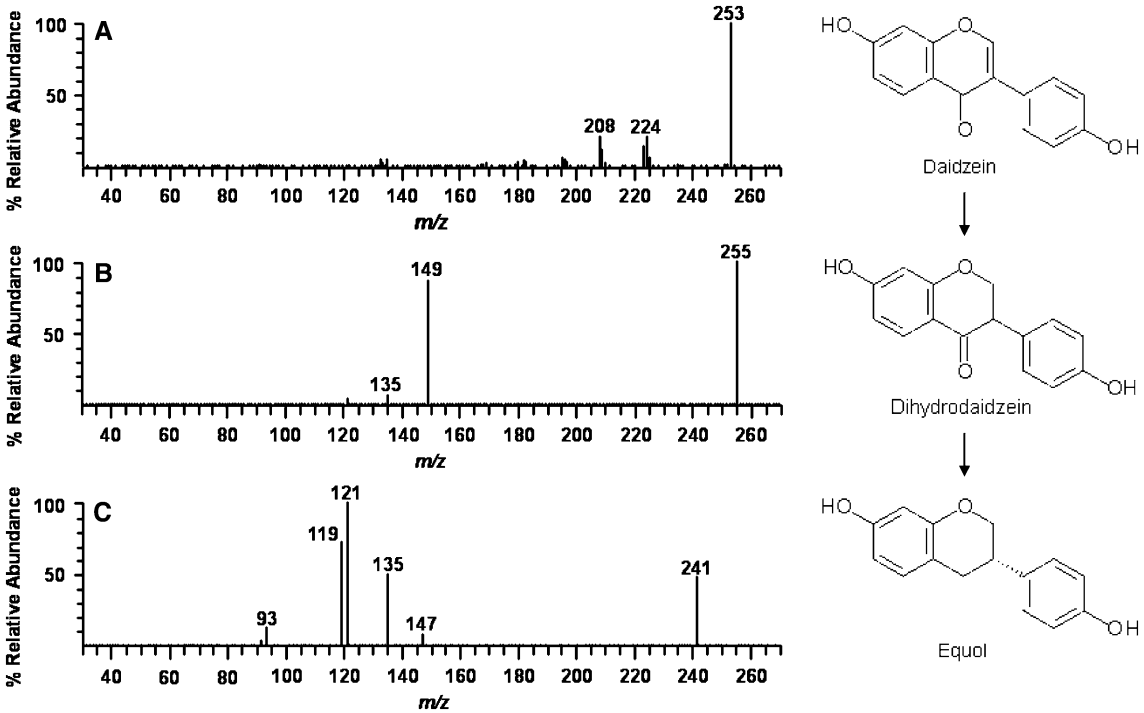

Fig. 2 Negative-ion electrospray product-ion spectra of daidzein and the metabolites produced during incubation with the monkey microbiota. **a** Daidzein; **b** dihydrodaidzein; and **c** equol. The product-ion spectra of the metabolites matched those of standards for dihydrodaidzein and equol

Different antibiotics have been shown to affect the urinary concentrations of isoflavones differently in adults and children [6, 8]. Three antibacterial agents affected daidzein metabolism by microbial cultures differently. Tetracycline inhibited daidzein-metabolizing bacteria and ciprofloxacin favored the population producing dihydrodaidzein. Ceftriaxone had no effect. The lack of population of bacteria metabolizing daidzein in tetracycline treated cultures in vitro in our study is similar to the finding that doxycycline, another tetracycline drug, inhibits production of equol in monkeys [1]. This indicates that the species of bacteria involved in daidzein metabolism are sensitive to the effects of tetracycline drugs. Ceftriaxone, a β-lactam antibiotic, had no effect on daidzein metabolism by the monkey microbiota. Ciprofloxacin, a fluoroquinolone, enhanced the metabolism of daidzein to dihydrodaidzein and equol in

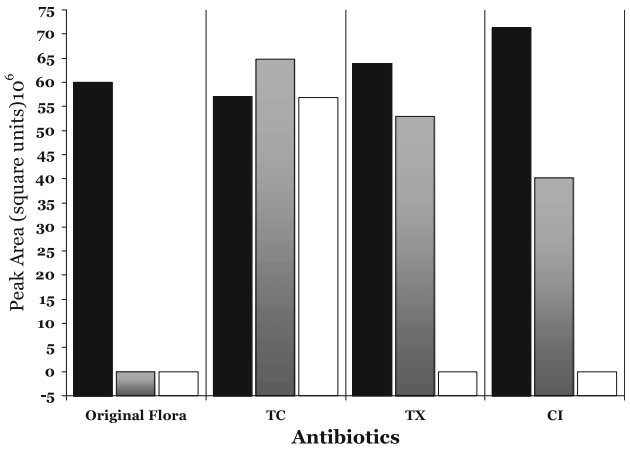

Fig. 3 Effect of antimicrobial agents on daidzein metabolism by cultures of the intestinal microflora of a monkey, as shown by HPLC. Each of the *bars* shows the amount of daidzein remaining in each of the cultures, treated with or without antibiotics, at the time designated. *Black bars* = 0 h, *gray bars* = 24 h, and *white bars* = 96 h. Original flora = cells obtained from a frozen sample of the colonic microflora; *TC* cells resistant to tetracycline, *TX* cells resistant to ceftriaxone, *CI* cells resistant to ciprofloxacin

mixed cultures, suggesting that ciprofloxacin removed the bacteria that are unable to metabolize daidzein. By eliminating these bacteria competing for nutrients, it enhanced the growth of the bacteria that convert daidzein to dihydrodaidzein and equol.

None of the individual bacteria isolated from the mixture of ciprofloxacin-resistant daidzein-metabolizing bacteria could metabolize daidzein. Although the microfloras of monkeys and humans metabolize daidzein [14, 15], in spite of efforts by our laboratory and others to detect specific bacteria involved in this process, only a few bacterial species from the intestinal microflora are reported to metabolize daidzein. It could be hypothesized either that bacteria metabolizing daidzein have not survived in monocultures or that the enzymes metabolizing daidzein in those bacteria are induced by factors produced by metabolic activities of other microorganisms. Bacterial consortia have been shown to overcome the limitations of individual species in the metabolism of other compounds [2].

In conclusion, by using Etest strips with several antibiotics to eliminate antibiotic-sensitive bacteria from a complex mixture of colonic microflora, we have been able to find out not only how antibiotic treatments affect daidzein metabolism but also how to enrich bacteria with daidzein metabolic capabilities from a complex colonic bacterial mixture. The microbiota of the intestinal tract is a complex ecosystem involved in beneficial metabolic activities, including conversion of drugs, nutrients, and food supplements. Although some individual strains of bacteria are capable of metabolizing specific compounds, the cooperation of groups of microorganisms may be responsible for some metabolic processes. This method, using multiple Etest strips, can be applied for selection of bacteria with specific metabolic capabilities from any complex microbiota.

Acknowledgments We offer special thanks to Dr. Anane Aidoo and Dr. Mugimane Manjanatha for reviewing the manuscript and Dr. Carl E. Cerniglia for his research support. This study was supported in part by an appointment (B.M.B.) to the Summer Student Research Program at the National Center for Toxicological Research, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration. The views presented in this article do not necessarily reflect those of the FDA.

References

1. Blair RM, Appt SE, Franke AA, Clarkson TB (2003) Treatment with antibiotics reduces plasma equol concentration in cynomolgus monkeys (*Macaca fascicularis*). J Nutr 133:2262–2267
2. Chatterjee S, Dutta TK (2008) Metabolic cooperation of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY in the utilization of butyl benzyl phthalate: effect of a novel co-culture in the degradation of a mixture of phthalates. Microbiology 154:3338–3346
3. Choi MS, Rhee KC (2006) Production and processing of soybeans and nutrition and safety of isoflavone and other soy products for human health. J Med Food 9:1–10
4. Claus SP, Ellero SL, Berger B, Krause L, Bruttin A, Molina J, Paris A, Want EJ, de Waziers I, Cloarec O, Richards SE, Wang Y, Dumas ME, Ross A, Rezzi S, Kochhar S, Van Bladeren P, Lindon JC, Holmes E, Nicholson JK (2011) Colonization-induced host-gut microbial metabolic interaction. mBio 2:1–10
5. Frank DN, Pace NR (2008) Gastrointestinal microbiology enters the metagenomics era. Curr Opin Gastroenterol 24:4–10
6. Franke AA, Halm BM, Ashburn LA (2008) Urinary isoflavones are increased in adults, but decreased in children, consuming soy when on oral antibiotic therapy. Nutr Cancer 60:627–635
7. Gardana C, Canzi E, Simonetti P (2009) The role of diet in the metabolism of daidzein by human faecal microbiota sampled from Italian volunteers. J Nutr Biochem 20:940–947
8. Halm BM, Ashburn LA, Franke AA (2007) Isoflavones from soya foods are more bioavailable in children than adults. Br J Nutr 98: 998–1005
9. Hur HG, Lay JO, Beger RD, Freeman JP, Rafii F (2000) Isolation of human intestinal bacteria metabolizing the natural isoflavone glycosides daidzin and genistin. Arch Microbiol 174:422–428
10. Hur HG, Beger RD, Heinze TM, Lay JO, Freeman JP, Dore J, Rafii F (2002) Isolation of an anaerobic intestinal bacterium capable of cleaving the C-ring of the isoflavonoid daidzein. Arch Microbiol 178:8–12
11. Jin MJ, Kim U, Kim IS, Kim Y, Kim DH, Han SB, Kwon OS, Yoo HH (2010) Effects of gut microflora on pharmacokinetics of hesperidin: a study on non-antibiotic and pseudo-germ-free rats. J Toxicol Environ Health 73:1441–1450
12. Jou HJ, Wu SC, Chang FW, Ling PY, Chu KS, Wu WH (2008) Effect of intestinal production of equol on menopausal symptoms in women treated with soy isoflavones. Int J Gynecol Obstet 102: 44–49
13. Magee PJ (2011) Is equol production beneficial to health? Proc Nutr Soc 70:10–18

14. Rafii F, Davis C, Park M, Heinze TM, Beger RD (2003) Variations in metabolism of the soy isoflavonoid daidzein by human intestinal microfloras from different individuals. Arch Microbiol 80:11–16
15. Rafii F, Hotchkiss C, Heinze TM, Park M (2004) Metabolism of daidzein by intestinal bacteria from rhesus monkeys (*Macaca mulatta*). Comp Med 54:165–169
16. Rafii F, Jackson LD, Ross I, Heinze TM, Lewis SM, Aidoo A, Lyn-Cook L, Manjanatha M (2007) Metabolism of daidzein by fecal bacteria in rats. Comp Med 57:282–286
17. Sekirov I, Tam NM, Jogova M, Robertson ML, Li Y, Lupp C, Finlay BB (2008) Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. Infect Immun 76:4726–4736
18. Tamura M, Hori S, Nakagawa H (2011) *Lactobacillus rhamnosus* JCM 2771: impact on metabolism of isoflavonoids in the fecal flora from a male equol producer. Curr Microbiol 62:1632–1637
19. Wähälä K, Salakka A, Adlercreutz H (1998) Synthesis of novel mammalian metabolites of the isoflavonoid phytoestrogens daidzein and genistein. Proc Soc Exp Biol Med 217:293–299
20. Wlodarska M, Willing B, Keeney KM, Menendez A, Bergstrom KS, Gill N, Russell SL, Vallance BA, Finlay BB (2011) Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated *Citrobacter rodentium*-induced colitis. Infect Immun 79:1536–1545
21. Yuan JP, Wang JH, Liu X (2007) Metabolism of dietary soy isoflavones to equol by human intestinal microflora–implications for health. Mol Nutr Food Res 51:765–781