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Metabolism of Daidzein by Intestinal Bacteria from Rhesus Monkeys (*Macaca mulatta*)

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Purpose: **To identify the metabolites produced from an isoflavonoid, daidzein, by colonic bacteria of rhesus monkeys.**

Methods: **The metabolism of daidzein by the fecal bacteria of nine monkeys was investigated. Daidzein was incubated anaerobically with fecal bacteria, and the metabolites were analyzed by use of liquid chromatography and mass spectrometry.**

Results: **The fecal bacteria of all of the monkeys metabolized daidzein to various extents. Dihydrodaidzein was found in cultures of fecal bacteria from two monkeys; dihydrodaidzein and equol were found in cultures from four monkeys; dihydrodaidzein, equol, and an unknown metabolite (MW = 244) were found in cultures from one monkey; and dihydrodaidzein and the unknown metabolite were found in cultures from two monkeys.**

Conclusions: **Similar to that in humans, variation was evident in the metabolism of isoflavonoids by fecal bacteria from rhesus monkeys. Some metabolites produced by fecal bacteria from monkeys were the same as those produced by fecal bacteria from humans.**

Isoflavonoids are diphenols that are found in leguminous plants, especially soybeans, and have natural roles in plant defense and root nodulation (22, 23). There is considerable interest in human consumption of soy isoflavonoids, which may decrease risk for cardiovascular diseases and hormone-related cancers (1, 18, 29, 34). Daidzein (4,7-dihydroxyisoflavone), a principal soy isoflavonoid, is a weakly estrogenic compound with potential health benefits for several conditions, including hypercholesterolemia and osteoporosis (1, 19, 20, 33). Isoflavonoids, including daidzein, may be converted to metabolites by intestinal microflora (5, 14, 25). This conversion is essential for absorption, bioavailability, and estrogenic activity of these compounds (16, 30, 35).

Monkeys and rodents have been used to predict the effects of phytoestrogens in humans (2, 9, 10, 34). One of the measurable factors that indicates the effectiveness of phytoestrogens is the impact on lipoprotein concentration. Low-density lipoprotein plus very low-density lipoprotein (LDL + VLDL) concentration is reduced by approximately 30%, and high-density lipoprotein (HDL) concentration is increased by approximately15% in the plasma of nonhuman primates after administration of dietary soy protein (3). Only modest changes in plasma lipoprotein concentration have been observed when soy/isoflavones have been administered to postmenopausal women in amounts comparable to those used in monkeys (2, 3, 17). Differences between plasma lipoprotein responses of monkeys and humans to soy products have been attributed in part to species differences in isoflavone metabolism (3). In general, 22 to 47% of human subjects consuming soy products have measurable quantities of equol (7-hydroxy-3-[4'-hydroxyphenyl]-chroman), a daidzein metabolite (3, 15). In contrast, nonhuman primates produce high amounts of equol (3). In postmenopausal cynomolgus monkeys fed soy diets, approximately 60% of the total plasma isoflavones concentration consists of equol (3, 6).

The effects of equol on health are difficult to evaluate due to variable equol production in human subjects consuming soy products (3). Equol is more estrogenic than is daidzein (27), has greater antioxidant activity than other isoflavones, and is circulated in higher amounts in the blood for a longer time (8, 15, 16, 21, 30, 32). Variability in equol excretion in humans has been attributed to differences in specific components of the gut microflora (4, 14, 15, 25). Blair and co-workers (3) used antibiotics to alter the intestinal bacteria in monkeys and investigated their effect on the plasma concentration of equol. Equol concentration was reduced after treatment with several antimicrobial agents, indirectly indicating the involvement of bacteria from the monkey intestinal flora in the metabolism of phytoestrogens. In the study reported here, the metabolism of daidzein by the intestinal bacteria in culture of feces from rhesus monkeys was investigated.

Materials and Methods

Animals. Nine rhesus monkeys (*Macaca mulatta*), aged two to 12 years, were studied (Table 1). The four females and five males were housed in single cages in an AAALAC-approved facility and were provided water ad libitum. They were fed High Protein Monkey Diet Jumbo (PMI Nutrition International, Richmond, Ind.) containing 25.0% protein, 5.0% fat, 5.0% fiber, and 49.7% carbohydrate. Primary diet components were derived from soybeans, wheat, and corn.

Metabolism of daidzein. Swab specimens were obtained from nine monkeys (Table 1) during fecal collection performed as part of the preventive medicine program for nonhuman primates. The swabs were transferred to tubes containing 10 ml of sterile

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Table 1. Sex and age of the monkeys of the study, and types of metabolites produced after incubation of fecal bacteria with daidzein

Monkey	Age	Sex	Metabolite produced		
			Dihydrodaidzein	Equol	Unknown (MW 244)
AR-182	12	M	+	+	...
AR-186	11	M	+	+	...
AR-187	11	M	+	+	...
AR-189	12	M	+	...	...
AR-289	12	F	+	...	...
AR-294	11	F	+	+	...
AR-316	3	M	+	...	+
AR-422	2	F	+	...	+
AR-425	5	F	+	+	+

(...) = Metabolites not found.

10% nonfat powdered milk in water, which was covered with two milliliters of sterilized mineral oil. After the tubes had been transferred to an anaerobic glove box (Forma Scientific, Marietta, Ohio), each swab was used to inoculate tubes containing brain heart infusion (BHI) medium under a $CO_2/H_2/N_2$ atmosphere (5:10:85, by volume). To one tube of medium from each set was added daidzein (5 µg/ml), then the cultures were incubated under $CO_2/H_2/N_2$ at 37°C (25). As a control, sterile BHI medium was incubated with 5 µg of daidzein/ml; 2.5 ml was removed aseptically from each of the cultures and controls every day and was extracted with ethyl acetate, which was dried over anhydrous Na_2SO_4 and evaporated. The residues were dissolved in 90% acetonitrile:10% water for high-performance liquid chromatography (HPLC) analysis (11-13). Cell density was measured spectrophotometrically.

The HPLC analysis. The Star HPLC system from Varian, Inc. (Palo Alto, Calif.) consisted of a model 230 pump, a model 430 autosampler with a 100-µl loop, and a model 330 photodiode array spectrophotometer. A Spherisorb C18 column (4.6 × 250 mm, S5, ODS 2 Phase Sep, Clwyd, Wales, UK) was used. The mobile phase components were 10% acetonitrile, 0.1% acetic acid, and 90% water (solution A) and 90% acetonitrile, 0.1% acetic acid, and 10% water (solution B). After sample injection, the column was washed with 100% solution A for 10 min, then the compounds were eluted, using a linear gradient of 10% solution B to 90% solution B for 50 min. The UV detector was monitored at 280 nm, and spectra of the peaks were scanned from 220 to 450 nm. The flow rate was 1 ml/min.

Liquid chromatography mass spectrometry (LC/MS). Analyses were performed, using a Hewlett-Packard 5989B (Palo Alto, Calif.) mass spectrometer with a Hewlett-Packard 1090L/M HPLC and a Prodigy ODS (3) 2.0 × 250 mm (5 µm, 100 Å) column (Phenomenex, Torrance, Calif.). The mass spectrometer was operated in the negative-ion electrospray ionization (ESI) mode, with the capillary exit voltage at –200 V for in-source collision-induced dissociation (CID). Full scans were acquired from m/z 50 to 300. The mobile phase, delivered at 0.2 ml/min, was a linear gradient from 20% aqueous acetonitrile to 80% aqueous acetonitrile for 40 min with constant 3 m*M* ammonium formate.

Chemicals. Daidzein and equol were purchased (Sigma Chemical Co., St Louis, Mo. and Indofine Chemical Co., Somerville, N.J., respectively). The HPLC-grade acetonitrile and methanol were from J. T. Baker (Phillipsburg, N.J.). Dihydrodaidzein was synthesized in our laboratory (11, 31).

Results

All cultures of monkey fecal flora grew equally well under anaerobic conditions, with or without 5 mg of daidzein/ml, as indicated by their optical density values. Daidzein and its metabolites, dihydrodaidzein and equol, eluted at different times and produced peaks with distinct UV spectra. The HPLC elution profile (data not shown) and LC/MS total ion chromatogram of a control, in which sterile daidzein was incubated in BHI medium, had only one peak representing daidzein, which was absent in the profiles obtained using BHI alone. The chromatograms of fecal cultures from various monkeys after incubation with and without daidzein were compared. The major peaks, other than that for daidzein, that were absent in cultures of intestinal bacteria without daidzein but present in cultures incubated with daidzein, were presumed to be daidzein metabolites (Fig. 1). This was verified by the characteristic UV spectra, which were similar to those of daidzein metabolites, dihydrodaidzein and equol. The identities of these peak were confirmed on the basis of results of mass spectral analyses of metabolites obtained by use of negative-ion electrospray mass spectra with CID and authentic standards for dihydrodaidzein and equol. Metabolite I (Fig. 2A), with mass spectrum similar to that of dihydrodaidzein [m/z 255 $(M\text{-}H)^-$, 149, 135, 121, 91], was found in all nine cultures. Metabolite II (Fig. 2B), with mass spectrum similar to that of equol [m/z 241 $(M\text{-}H)^-$, 147, 135, 121, 119, 93], was found in five cultures. Metabolite III, found in three cultures, eluted later than equol, but

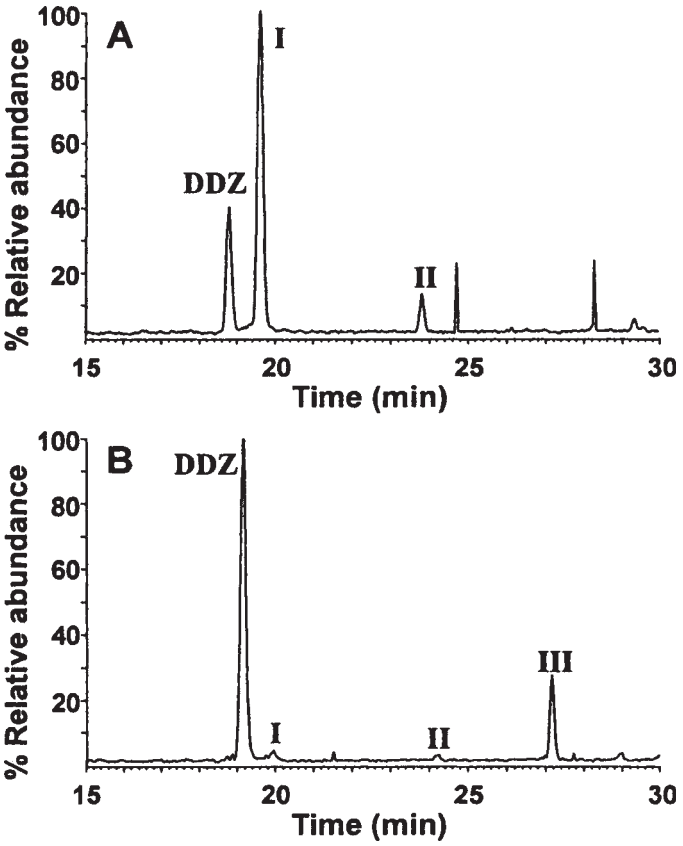

Figure 1. Total ion content after liquid chromatography/electrospray ionization (LC/ESI) of extracts containing daidzein from brain heart infusion medium cultures of intestinal microflora from two monkeys. The peaks unrelated to daidzein are not marked. DDZ = daidzein, peak I = dihydrodaidzein, peak II = equol, and peak III = unknown metabolite with molecular weight of 244. (A) Metabolites produced from monkey AR-186. (B) Metabolites produced from monkey AR-425. The profiles of extracts obtained from other monkeys are omitted for brevity.

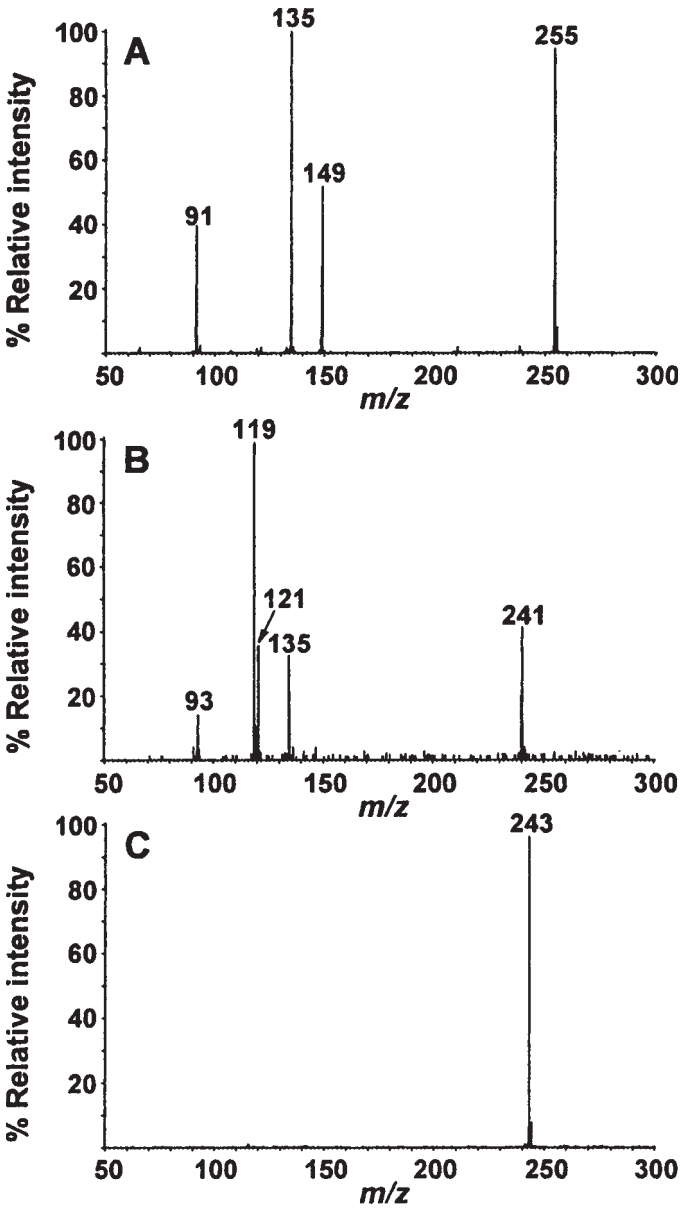

Figure 2. Electrospray mass spectra obtained by use of collision-induced dissociation (CID), of daidzein metabolites produced by the microfloras of various individual monkeys. Dihydrodaidzein (A, metabolite I), equol (B, metabolite II), and unknown metabolite of molecular weight 244 (C, metabolite III). Metabolites I and II were compared with standards (not shown). Metabolite III did not fragment.

had a UV spectrum similar to that of equol. Its mass spectrum (Fig. 2C) had an ion at m/z 243 $(M\text{-}H)^-$, indicating molecular weight of 244. The ion at m/z 243 did not fragment at –200 V, which usually induces fragmentation. This metabolite, which was not found in any of the cultures without daidzein, was designated as an unknown (MW 244). It was found with dihydrodaidzein in two cultures, and with dihydrodaidzein and equol in one culture. The metabolites from the various cultures are shown in Table 1.

Discussion

Monkeys have been used to study the effect of phytoestrogens on prevention of hormone-dependent diseases in humans (2, 3, 6). One of the beneficial properties of some phytoestrogens is a plasma cholesterol-lowering effect, which should provide protection against coronary artery atherosclerosis (2, 29). However, despite achieving major reductions in plasma LDL+VLDL values and increases in HDL concentration in nonhuman primates, administration of dietary soy products in human subjects results in only small changes in lipoproteins (17). Furthermore, higher amounts of equol, a daidzein metabolite, have been produced in monkeys, compared with human subjects, after soy product administration (3). These findings have been attributed to differences in the metabolism of isoflavonoids by the respective microflora of monkeys and humans (3).

Administration of several antimicrobial agents to monkeys has been correlated with decreases in equol production, suggesting the involvement of colonic microflora in daidzein metabolism (3). In the study reported here, the ability of fecal bacteria from rhesus monkeys to metabolize daidzein has been documented.

Dihydrodaidzein and equol were the two principal daidzein metabolites produced by the fecal flora and were found in five of nine cultures (Fig. 3). We previously reported that dihydrodaidzein, equol, and *O*-desmethylangolensin (*O*-DMA) were produced during incubation of intestinal microflora of some human subjects consuming soy products (25). The *O*-DMA, which is produced by ring cleavage of dihydrodaidzein, has been found in human urine after consumption of soy isoflavones (14, 15) following incubation of daidzein with fecal bacteria from humans (12, 28), and in urine of rat with human colonic microflora (4). It was not found in any of the monkey fecal cultures.

Clarkson and co-workers (6) found less *O*-DMA (18 ± 2 U) than equol (459 ± 45 U) in the plasma of cynomolgus monkeys treated with soy phytoestrogens. Since dihydrodaidzein is an intermediate in the conversion of daidzein to equol and *O*-DMA (12), perhaps the populations of bacteria producing equol are appreciably higher in monkeys than in humans. Equol, a nonsteroidal estrogen, is more estrogenic than daidzein. It is a product of the intestinal bacterial metabolism of daidzein (4, 25) attributed to the beneficial effect of soy products (4, 14, 24, 30). The effectiveness of soy protein in cardiovascular, bone, and menopausal health may be a function of the ability of the intestinal microbiota to biotransform soy isoflavones to the more potent estrogenic isoflavone, equol (12). However, equol is not produced in all

Figure 3. Proposed pathway of daidzein metabolism by fecal microflora as suggested by Joannou and co-workers (14). Intermediate compounds that have been suggested to lead to the production of equol were not found.

healthy adults in response to the consumption of soy products or daidzein (4, 14, 25). Species differences in soy isoflavone metabolism may result in differences between plasma lipoprotein concentrations of monkeys and human subjects (3). In this regard, lack of production of *O*-DMA by the fecal microflora of rhesus monkeys correlates with the minor amount of *O*-DMA produced in cynomolgus monkeys (6). Regardless of sex, equol was not found in four of the cultures; thus, differences in the prevalence of bacteria producing equol may exist among monkeys.

There appears to be a correlation between the age of the monkeys and the type of metabolites produced. The unknown metabolite (MW 244) was found only in the younger monkeys (two to five years old), which could be attributed to differences between the microflora of these animals and those of older monkeys (7). The principal dietary components of these monkeys were derived from soybeans, wheat, and corn. Therefore these monkeys were exposed to phytoestrogens through the diet. The metabolism of phytoestrogens by intestinal bacteria should be considered when the data obtained from the effects of phytoestrogens are evaluated (3, 30).

Blair and co-workers (3) determined that treatment with antibiotics reduces plasma equol concentration in cynomolgus monkeys. In the study reported here, we found that intestinal microflora of monkeys produced some of the same metabolites as did human intestinal bacteria. However, nonestrogenic metabolites, such as *O*-DMA, which reduces the effectiveness of phytoestrogens (14), were not found. The amount of *O*-DMA found in the plasma of cynomolgus monkeys treated with soy phytoestrogens was three percent of the amount of equol (6), which could have been produced by the intestinal microflora or other enzymes. Although equol was found in the cultures after two days of incubation, longer incubation of daidzein with fecal flora did not result in *O*-DMA production. The conditions in this study were the same as those used for the detection of metabolites produced by the human fecal flora, in which *O*-DMA was detected.

The unknown compound, with a UV spectrum similar to that of equol and molecular weight of 244, needs further characterization. All monkeys used in this study were fed the same diet, and although they were housed singly, they were in close proximity, which increases the possibility of similar intestinal microflora. However, we found differences in the metabolism of daidzein by these cultures from these monkeys. The variability in the metabolism of phytoestrogens in humans may be greater because of differences in diet and environmental exposure (26). The lack of a controlled environment for humans must also be considered when extrapolating results from laboratory animals.

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References

1. **Adlercreutz, H.** 1995. Phytoestrogens: epidemiology and a possible role in cancer protection. Environ. Health. Perspect. **103 Suppl 7:**103-112.
2. **Anthony, M. S., T. B. Clarkson, B. C. Bullock, and J. D. Wagner.** 1997. Soy protein versus soy phytoestrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys. Arterioscler. Thromb. Vasc. Biol. **17:**2524-2531.
3. **Blair, R. M., S. E. Appt, A. A. Franke, and T. B. Clarkson.** 2003. Treatment with antibiotics reduces plasma equol concentration in cynomolgus monkeys (*Macaca fascicularis*). J. Nutr. **133:**2262-2267.
4. **Bowey, E., H. Adlercreutz, and I. Rowland.** 2003. Metabolism of isoflavones and lignans by the gut microflora: a study in germfree and human flora associated rats. Food Chem. Toxicol. **41:**631-636.
5. **Chang, Y.-C. and M. G. Nair.** 1995. Metabolism of daidzein and genistein by intestinal bacteria. J. Natl. Prod. **58:**1892-1896.
6. **Clarkson, T. B., M. S. Anthony, and T. M. Morgan.** 2001. Inhibition of postmenopausal atherosclerosis progression: a comparison of the effect of conjugated estrogens and soy progesterone. J. Clin. Endocrinol. Metab. **86:**41-47.
7. **Conway, P. L.** 1995. Microbial ecology of the human large intestine, p. 1-24. *In* G. R. Gibson and G. T. Macfarlane (ed.), Human colonic bacteria: role in nutrition, physiology and pathology, CRC Press, Boca Raton, Fla.
8. **Duncan, A. M., B. E. Merz-Demlow, X. Xu, W. R. Phipps, and M. S. Kurzer.** 2000. Premenopausal equol excretors show plasma hormone profiles associated with lowered risk of breast cancer. Cancer Epidemiol. Biomarkers Prevent. **9:**581-586.
9. **Fang, N., S. Yu, and T. M. Badger.** 2002. Characterization of isoflavones and their conjugates in female rat urine using LC/MS/MS. J. Agric. Food Chem. **50:**2700-2707.
10. **Harrison, R. M., P. P. Phillippi, K. F. Swan, and M. C. Henson.** 1999. Effect of genistein on steroid hormone production in the pregnant rhesus monkey. Proc. Soc. Exp. Biol. Med. **222:**78-84.
11. **Hur, H.-G., J. O. Lay, Jr., R. D. Beger, J. P. Freeman, and F. Rafii.** 2000. Isolation of human intestinal bacteria metabolizing the natural isoflavone glycosides daidzin and genistin. Arch. Microbiol. **174:**422-428.
12. **Hur, H.-G., R. D. Beger, T. M. Heinze, J. O. Lay, J. P. Freeman, J. Dore, and F. Rafii.** 2002. Isolation of an anaerobic intestinal bacterium capable of cleaving the C-ring of the isoflavonoid daidzein. Arch. Microbiol. **178:**8-12.
13. **Hur, H.-G. and F. Rafii.** 2000. Biotransformation of the isoflavonoids biochanin A, formononetin and glycitein by *Eubacterium limosum*. FEMS Microbiol. Lett. **192:**21-25.
14. **Joannou, G. E., G. E. Kelly, A. Y. Reeder, M. Waring, and C. Nelson.** 1995. A urinary profile study of dietary phytoestrogens. The identification and mode of metabolism of new isoflavonoids. J. Steroid Biochem. Mol. Biol. **54:**167-184.
15. **Kelly, G. E., G. E. Joannou, A. Y. Reeder, C. Nelson, and M. A. Waring.** 1995. The variable metabolic response to dietary isoflavones in humans. Proc. Soc. Exp. Biol. Med. **208:**40-43.
16. **Lampe, J. W., H. E. Skor, S. Li, K. Wähälä, W. N. Howald, and C. Chen.** 2001. Wheat bran and soy protein feeding do not alter urinary excretion of the isoflavan equol in premenopausal women. J. Nutr. **131:**740-744.
17. **Lichtenstein, A. H., S. M. Jalbert, H. Adlercreutz, B. R. Golden, H. Rasmussen, E. J. Schaefer, and L. M. Ausman.** 2002. Lipoprotein response to diet high in soy or animal protein without and with isoflavones in moderately hypercholesterolemic subjects. Arterioscler. Thromb. Vasc. Biol. **22:**1852-1858.
18. **Messina, M. and J. Erdman.** 2000. Third International Symposium on the role of soy in preventing and treating chronic disease. J. Nutr. **130:**653S.
19. **Messina, M.** 2000. Soyfoods and soybean phyto-oestrogens (isoflavones) as possible alternatives to hormone replacement therapy (HRT). Eur. J. Cancer **36:**71-72.
20. **Messina, M. and V. Messina.** 2000. Soyfoods, soybean isoflavones, and bone health: a brief overview. J. Renal Nutr. **10:**63-68.
21. **Nagel, S. C. V., F. S. Van Saal, and W. V. Welshone.** 1998. The effective free fraction of estradiol and xenoestrogens in human serum measured by the whole cell uptake assays: physiology of delivery modifies estrogenic activity. Proc. Soc. Exp. Biol. Med. **217:**300-309.

22. **Peters, N. K., J. N. Frost, and S. R. Long.** 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233:**977-980.
23. **Phillips, D. A.** 1992. Flavonoids: plant signals to soil microbes. Annu. Rev. Phytochem. **26:**201-231.
24. **Rafii, F.** 1998. Modification of biological activities of phytoestrogens by intestinal microflora. Recent Res. Dev. Agric. Food Chem. **2:**803-808.
25. **Rafii, F., C. Davis, M. Park, T. M. Heinze, and R. D. Beger.** 2003. Variation in metabolism of the soy isoflavonoid daidzein by human intestinal microfloras from different individuals. Arch. Microbiol. **180:**11-16.
26. **Rowland, I. R., H. Wiseman, T. A. Sanders, H. Adlercreutz, and E. A. Bowey.** 2000. Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. Nutr. Cancer **36:**27-32.
27. **Sathyamoorthy, N. and T. T. Wang.** 1997. Differential effects of dietary phyto-oestrogens daidzein and equol on human breast cancer MCF-7 cells. Eur. J. Cancer **33:**2384-2389.
28. **Schoefer, L., R. A. Mohan, M. Braune, M. Birringer, and M. Blaut.** 2002. Anaerobic C-ring cleavage of genistein and daidzein. FEMS Microbiol. Lett. **208:** 197-202.
29. **Setchell, K. D. R.** 1995. Phytoestrogens: epidemiology and possible role in cancer protection. Environ. Health Perspect. **103:**103-112.
30. **Setchell, K. D. R., N. M. Brown, and E. Lydeking-Olsen.** 2002. The clinical importance of the metabolite equol—a clue to the effectiveness of soy and isoflavones. J. Nutr. **132:**3577-3584.
31. **Wähälä, K., A. Salakka, and H. Adlercreutz.** 1998. Synthesis of novel mammalian metabolites of the isoflavonoid phytoestrogens daidzein and genistein. Proc. Soc. Exp. Biol. Med. **217:**293-299.
32. **Watanabe, S., M. Yamaguchi, T. Sobue, T. Takahashi, T. Miura, Y. Arai, W. Mazur, K. Wähälä, and H. Adlercreutz.** 1998. Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako). J. Nutr.**128:**1710-1715.
33. **Wilcox, G., M. L. Wahlqvist, H. G. Burger and G. Medley.** 1990. Oestrogenic effects of plant foods in postmenopausal women. Br. Med. J. **301:**905-906.
34. **Williams, J. K., M. S. Anthony, and D. M. Herrington.** 2001. Interactive effect of soy protein and estradiol on coronary artery reactivity in atherosclerotic, ovariectomized monkeys. Menopause **8:**307-313.
35. **Xu, X., K. S. Harris, H. J. Wang, P. A. Murphy, and S. Hendrich.** 1995. Bioavailability of soybean isoflavones depends upon gut microflora in women. J. Nutr. **125:**2307-2315.