Human gut microfloral metabolism of soybean isoflavones

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Toxicology
Major Professor: Suzanne Hendrich

Iowa State University
Ames, Iowa
1997

Graduate College

Iowa State University

This is to certify that the Master thesis of

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INTRODUCTION

Isoflavones are diphenolic phytoestrogens found primarily in legumes. The predominant isoflavones found in soybeans are daidzin, genistin, glycitin and their corresponding aglycones daidzein, genistein and glycitein (Murphy, 1982). Recently, increasing evidence have associated soybean isoflavones with some health beneficial effects. Epidemiological studies show soybean consumption may contribute to the lower rates of sex-hormone dependent cancers (such as breast, colon, and prostate cancers) in Asian countries (Adlercreutz et al., 1991; Rose et al., 1986; Muri et al., 1987). Urinary excretion of isoflavones and their metabolites is substantially greater in Japanese than in people living in Western countries (Adlercreutz et al., 1991). Compared with healthy controls, breast cancer patients excrete about 21% less of the isoflavone metabolite equol (Adlercreutz et al., 1982) in urine. Genistein, a major isoflavone, suppresses mammary cancer in a dimethylbenz [α] anthracene (DMBA) initiated rat model in vivo. Isoflavones are also found to suppress early liver cancer promotion by phenobarbital in diethylnitrosamine (DEN) initiated rats as determined by measuring hepatic glutathione peroxidase activity and quantifying liver glutamyltransferase (GGT)-positive (GGT^+) and placental glutathione transferase (PGST)-positive ($PGST^+$) altered hepatic foci (AHF) (Lee et al., 1995). Furthermore, genistein and daidzein inhibits the growth of human breast cells, both estrogen receptor-positive (MCF-7) and -negative (MDA-468) cell lines (Peterson and Barnes, 1991). The concentrations needed to inhibit 50% of cell growth are 24-44 μmol/L and 79-134 μmol/L for genistein and daidzein, respectively. Genistein may also block mammary epithelial cell growth by

modulating signal transduction events stimulated by estradiol or growth factors (Peterson and Barnes, 1996). Estradiol activates human colon carcinoma-derived Caco-2 cell proliferation by stimulating four signaling transduction proteins. Genistein, a tyrosine kinase inhibitor, abolishes the estradiol stimulatory effect on Caco-2 cell proliferation (Demenico et al., 1996).

Phytoestrogenic isoflavones posses antiestrogenic activity which is a possible mechanism of their anticarcinogenic activities. Genistein and daidzein compete with estradiol for estrogen receptor (E_2 receptor) binding (Newsome and Kitts, 1980; Farmakalidis et al., 1985), but after binding, those phytoestrogens are much less effective than 17β-estradiol and synthetic estrogen diethylstilbestrol (DES) in translocating the binding complex to the nucleus and stimulating the synthesis of more receptors (Folman and Pope 1969; Martin et al., 1978). Isoflavones may also exert their antiestrogenic effects indirectly by increasing liver synthesis of sex-hormone binding globulin (SHBG; Adlercreutz et al., 1987; Adlercreutz et al., 1992) and inhibiting aromatase activities (Adlercreutz et al., 1993). The antiestrogenic effects of soybean isoflavones may be beneficial to protect sex-hormone dependent cancers.

Isoflavones may also exert some of their anticarcinogenic effects through an antioxidant mechanism. Polyphenolic compounds in soybeans have antioxidant activity detected by measuring the rate of β-carotene bleaching in a lipid-aqueous system (Pratt and Birac, 1979). Genistein decreases the production of reactive oxygen species (ROS) by tumor cell types and cells of the immune system (Wei et al., 1993; Akimura et al., 1987; Naim et

al., 1976). Feeding one week of soy isoflavone extract at 240 mg isoflavones /kg diet significantly increases rat hepatic glutathione peroxidase activity (Hendrich et al., 1994). Soy isoflavone extract is shown to normalize rat liver cytosolic glutathione S-transferase activity elevated by Phenobarbital (Lee et al., 1995).

In addition to their anticacinogenic activities, isoflavones have other health beneficial effects. Isoflavones are found to improve cardiovascular risk factors (Anthony et al., 1996). Compared with soy protein depleted of isoflavones (removed by alcohol extraction), isoflavone containing soy protein significantly decreases plasma LDL and VLDL cholesterol concentrations in both female and male rhesus monkeys after 6 months of feeding. In a three-week feeding study, soy protein (180 g/kg diet) suppresses hepatic lipogenic enzyme (acetyl-CoA carboxylase, fatty acid synthase, malic enzyme, and ATP-citrate lyase) gene expression in Wistar fatty rats when compared to casein diet. Furthermore, soy protein has beneficial effects on bone metabolism. Soy protein isolate prevents vertebral and femoral bone mass loss in ovariectomized rats compared to casein diet fed three months (Arjmandi et al., 1996). Rats fed 28 days of soymilk in addition to dietary casein have a higher intestinal calcium absorption rate and greater lumbar spine and tibia bone mineral density compare with casein only fed rats (Omi et al., 1994).

Recently, the health beneficial effects of soybean isoflavones have prompted researchers to study the bioavailability and metabolism of those compounds. The absorption of isoflavones is dose dependent (Xu et al., 1994). The 24 hr urinary excretion of daidzein and genistein increased with doses of 0.7 to 1.3 and 2 mg total isoflavone per kg of body

weight from 5.93 ± 3.47 to 14.87 ± 7.65 and 20.26 ± 10.33 mg, respectively. Fecal excretion increases accordingly. At 6.5 hr post dosing, plasma total isoflavones are 1.53 ± 0.47, 2.29 ± 1.30, and 4.39 ± 2.50 μmol/L at doses of 0.7, 1.3, and 2 mg/kg body weight, respectively. The bioavailability of isoflavones is not affected by types of soybean food or the selection of diets (Xu, 1995). The 48 hr urinary excretion of daidzein (27 ± 8%, 26 ± 10%, and 26 ± 9%, respectively) or genistein (20 ± 7%, 18 ± 9%, and 18 ± 8%, respectively) are not affected by basic, self-selected, or ad libitum diets. Similarly, 24 hr urinary excretion of daidzein after consumption of cooked soybean, texturized vegetable protein, tofu, and tempeh are 46 ± 16%, 51 ± 10%, 50 ± 10%, and 38 ± 18%, respectively, and of genistein, 13 ± 6%, 14 ± 8%, 16 ± 5%, and 9 ± 4%, respectively. Furthermore, wheat fiber decreases the bioavailability of genistein (Tew et al., 1995). A supplement of 25 g wheat fiber to a 15 g fiber diet decreases genistein urinary excretion by 20%, whereas daidzein bioavailability is not affected.

The excretion of isoflavones is about 30% in urine and 1-2% in feces, respectively. Approximately 70% of the consumed isoflavones are not recoverable. In addition, individual bioavailability of isoflavones varies considerably among populations (Xu, et al., 1995). Three levels of isoflavones, 3.4, 6.9 and 10.3 μmol/kg body weight were given to seven women subjects on three test days each dose separated by 2 weeks of wash out period. Each dose level was consumed three times on the feeding day. Fecal genistein and daidzein excretion of two subjects were significantly higher than that of the other five subjects. Urinary isoflavone excretion of the two subjects excreting a large amount of fecal isoflavones were three times and two times higher than that of subjects excreting a small

amount of fecal isoflavones for genistein and daidzein, respectively. Plasma genistein concentration of the high isoflavone excretors was significantly higher than that of the low isoflavone excretors at 24 hr after dosing. Whereas, plasma daidzein concentrations of the two levels of excretors were not different at 6.5 and 24 hr post-dosing. Xu and his co-workers further showed that isoflavones were rapidly metabolized to unknown compounds by human gut microflora in an in vitro incubation of daidzein and genistein with human feces.

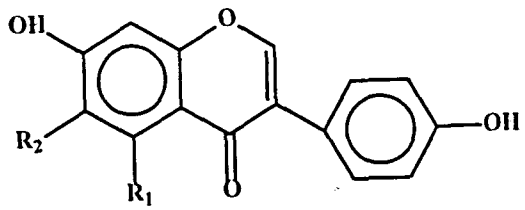
Based on the observation of Xu and his co-workers (1995), we hypothesized that gut microflora may have great impact on the bioavailability of isoflavones and the action of gut microflora on isoflavones may partially contribute to the observed great inter-individual variation of isoflavone bioavailability.

LITERATURE REVIEW

Isoflavones are phenolic compounds produced by plants. Soybeans are a major source of isoflavones (1-3 mg/g; Wang and Murphy, 1994a). The principal isoflavones found in soybeans fit into four chemical classes, aglycones (daidzein, genistein, glycitein), glucosides (daidzin, genistin, glycitin), acetylglucosides (6"-O-acetyldaidzin, 6"-O-acetylgenistin, 6"-O-acetylglycitin), and malonylglucosides (6"-O-malonyldaidzin, 6"-O-malonylgenistin, 6"-O-malonylglycitin) (Figure 1).The isoflavone contents of soybeans vary significantly according to the variety, year of harvest and location (Wang and Murphy, 1994b).The distribution of the 12 isoflavone isomers in commercial soybean foods are quite different (Wang and Murphy 1994a). Compared with unprocessed soybeans, high-protein soybean ingredients contain similar concentrations of isoflavones (1.2 - 4.2 mg/g), except alcohol-leached soybean concentrate. Unfermented soybean foods have greater levels of glucosides, whereas greater levels of aglycones are found in fermented soybean products, such as miso. Second-generation soybean foods such as tofu, yogurt and tempeh burgers contain only 6 - 20% of the isoflavones of whole soybean.

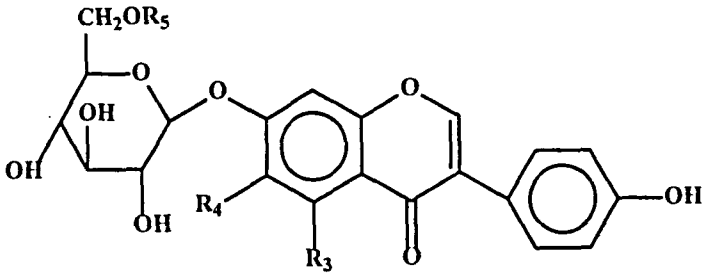
Soybean consumption is higher in Asian countries where the incidence of sex-hormone dependent cancers are low compared with people living in the Western world. Epidemiological studies have shown that larger amount of soybean product consumption may be associated with low incidence of hormone-dependent cancers, such as breast, colon and prostate cancer (Adlercreutz et al., 1991). Due to the structural similarity of isoflavones to endogenous estrogen 17β-estradiol, isoflavones may exert either estrogenic effects or

Aglycones



R_1	R_2	Compounds	Molecular Weight
H	H	Daidzein	254
OH	H	Genistein	270
H	OCH_3	Glycitein	284

Glucosides



R_3	R_4	R_5	Compounds	Molecular Weight
H	H	H	Daidzin	416
OH	H	H	Genistin	432
H	OCH_3	H	Glycitin	446
H	H	$COCH_3$	6"-O-Acetyldaidzin	458
OH	H	$COCH_3$	6"-O-Acetylgenistin	474
H	OCH_3	$COCH_3$	6"-O-Acetylglycitin	488
H	H	$COCH_2COOH$	6"-O-Malonyldaidzin	522
OH	H	$COCH_2COOH$	6"-O-Malonylgenistin	518
H	OCH_3	$COCH_2COOH$	6"-O-Malonylglycitin	532

Figure 1. Chemical structure of 12 isoflavone isomers that normally found in soy foods (Adapted from Wang and Murphy, 1994a).

antiestrogenic effects in vivo and in vitro depending on the circulating levels of isoflavones and species, tissues and cell lines under investigation (Bennets et al., 1946; Price and Fenwick, 1985; Wang et al., 1996). Isoflavones also possess antioxidant (Pratt and Birac, 1979; Naim, 1976) and antifungal activities (Naim, 1974). Moreover, isoflavones inhibit the growth of human breast cancer cell lines (Peterson and Barnes, 1991; Akiyama et al., 1987). Isoflavones also inhibit tyrosine-specific protein kinase and topoisomerase activities (Okura et al., 1988), two functions related to anticancer potential.

Epidemiological studies associated with soy consumption and cancer

The incidence of and mortality from breast and prostate cancer in countries of the Western world is significant greater than that in Asian countries, where the incidence of developing the diseases is 5-8 fold less (Rose et al., 1986, Muri et al., 1987). However, when Asian women immigrated to the US and gradually adapted to the western diet, the incidence of breast cancer and prostate cancer increased to the same rate as indigenous Westerners (Rose et al., 1986; Muri et al., 1987) suggesting that environmental factors, probably diet, have important roles in the development of sex-hormone dependent cancers.

Compared to the high fat, high protein, high calories, and low fiber western diet, the Oriental population consumes a rather low fat, low protein, low calories, and high fiber diet which partly contributes to the low rate cancer risk in those countires. Another great difference between the two diets is that soybean consumption is significantly greater in Asian countries than in Western countries (Adlercreutz et al., 1991). British intake of soybeans isoflavones has been estimated to be < 1 mg/d (Jones et al., 1989), while the

average consumption of these compounds in Asian countries is 50-100 mg/d (Barnes et al., 1990; Adlercreutz et al., 1991). Japanese men and women consuming a traditional diet excrete substantially greater amounts of isoflavones in urine than people living in Boston and Helsinki. Breast cancer patients excrete about 21% less of the isoflavone metabolite, equol, than healthy controls (Adlercreutz et al., 1982). Plasma isoflavone levels are 10-20 times higher in Japanese men than in Finnish males (Adlercreutz et al., 1993). In addition, Adlercreutz et al. (1995) showed that fecal excretion of plytoestrogens is substantially higher in vegetarian subjects than in a group of omnivores.

Several epidemiological trials have tested the hypothesis that the differences in hormone-dependent cancer incidence rates between Asian and Western populations may be associated with the action of phytoestrogens as protective agents in their diet. (Nomura et al., 1978; Severson et al., 1989). They found that the incidence of breast cancer was inversely correlated with soybean products consumption. Lee et al. (1991) performed a case-control study of diet and breast cancer in Chinese women in Singapore. They found that soy protein had a protective effect in premenopausal women and suggested that phytoestrogenic isoflavones in soybeans may be responsible for this protective effect.

Anticarcinogenic effects of isoflavones

Animal studies show a correlation between soybean products consumption and reduced cancer rates. Barnes et al. (1990) conducted an experiment to determine the effect of soybean diets on breast cancer tumorigenesis. Compared with casein diet, 20% powdered soybean chips reduced tumor load from 8 to 3 in the N-methylnitrosourea (NMU) induced

mammary cancer model in female rats. Hawrylewicz et al. (1991) also noticed that soybean protein isolate decreased tumors by 50% in an NMU model of breast cancer. Troll et al. (1980) showed a 50% reduction in X-ray irradiated mammary tumors in rats consuming a powdered soybean diet. When the isoflavones were chemically removed from the soybean product, no reduction in mammary carcinogenesis was observed in rats (Barnes et al., 1990). In a diethylnitrosamine (DEN) initiated rat liver cancer study, Lee et al. (1995) showed that soybean isoflavone extract suppressed early but not later promotion of hepatocarcinogenesis by phenobarbital in female rats. At doses of 920 and 1840 μmol total isoflavones /kg diet, soy isoflavone extract normalized total hepatic glutathione peroxidase activity and suppressed phenobarbital (PB) of hepatocarcinogenesis be decreasing the liver volume occupied by γ-glutamyltransferase-positive (GGT^+) and placental glutathione transferase-positive ($PGST^+$) altered hepatic foci (AHF) after 3 months of feeding. After 11 months of PB promotion, the low dose soy extract still had protective effect by decreasing $PGST^+$ AHF. However, GGT^+ AHF were not decreased by either dose of soy isoflavone extract after 11 months of PB promotion. Lamartiniere et al. (1995) showed that administration of genistein to rats early in life suppressed mammary cancer induced by dimethylbenz (α) anthracene (DMBA) in rats.

Genistein inhibits the growth of both estrogen receptor positive (ER^+)and negative (ER^-) human cancer cell lines (Peterson and Barnes, 1991). The same authors later showed that genistein inhibited both estrogen and growth factor stimulated proliferation of human breast cancer cell lines (MCF-7, T47D ER^+, T47D ER^-, BT-20, and ZR-75-1; Peterson and

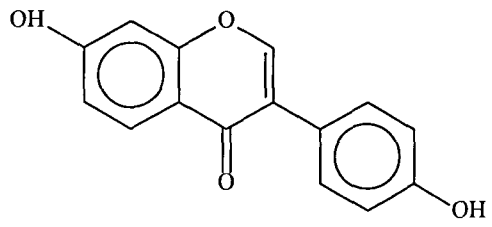
Barnes, 1996). The genistein concentrations needed to inhibit 50% of cancer cell growth (IC_{50}) range from 9.6 to over 74 μmol/L. The growth inhibition effect of genistein did not depend on the present of functional estrogen receptor or on the inhibition of epithelial growth factor (EGF) protein tyrosine kinase activity. They suggested that genistein may block mammary epithelial cell growth by interfering with signal transduction events stimulated by estradiol or growth factors. Genistein also blocks in vitro human colon cancer cell proliferation stimulated by estradiol (Domenico et al., 1996). Estradiol stimulates cell proliferation by triggering a tyrosine-specific protein kinase, erk-2. Genistein, a tyrosine kinase inhibitor, abolishes the estradiol stimulatory effect on both erk-2 activity and cell proliferation.

Furthermore, genistein and daidzein inhibit the production of inositol phosphates, key intracellular signals of proliferation stimulated by aluminum tetrafluoride in 3T3 cells (Higashi and Oganara, 1992). Genistein also blocks endothelial cell proliferation and in vitro angiogenesis, another proposed mechanism of anticarcinogenesis (Fotsis et al., 1993). In addition, isoflavones may exert their anticarcinogenic effects by inhibiting the activity of DNA topoisomerase I and II and modulating other enzymes involved in DNA synthesis (Okura, 1988).

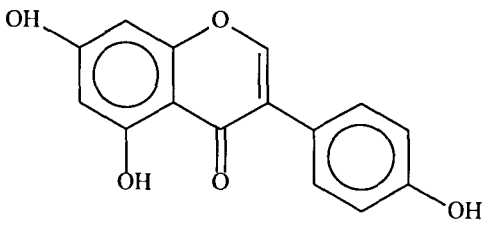
Estrogenic and antiestrogenic properties of isoflavones

Isoflavones are heterocyclic phenols closely related in structure to mammalian estrogen, 17β-estradiol. The distance between the two hydroxyl groups at the opposite poles

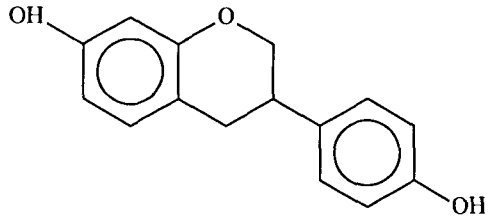
(1) Daidzein



(2) Genistein



(3) Equol



(4) 17-β estradiol

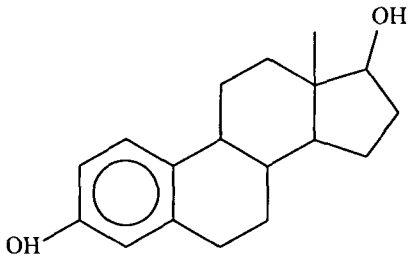


Figure 2. Chemical structures of isoflavone daidzein (1), genistein (2), daidzein metabolite equol (3), and endogenous estrogen 17-β estradiol (4).

of the molecules is similar to the distance between the C-3 and C-17 hydroxyl groups of 17β-estradiol, a prerequisite in binding to estrogen receptors (Figure 2). Weak estrogenic effects of isoflavones have been extensively reviewed by Moltteni (1995). Estrogenicity of genistein and daidzein are roughly 10^3 to 10^5 times lower than estradiol or the synthetic estrogen diethylstilbestrol (DES) (Farmakalidis et al., 1985; Kitts et al., 1980; Newsome and Kitts, 1980). Shutt and Cox (1972) reported that the binding affinity of genistein and daidzein to sheep uterine estrogen receptors are 0.9% and 0.1% that of 17β-estradiol, respectively. However, at higher concentrations such as 10^2 - 10^3 times of estradiol (close to the levels of isoflavones found in human plasma after soybean products consumption), these isoflavones could effectively compete with the endogenous estrogens by competitive binding to estrogen receptors and suppressing estrogen stimulated cell proliferation (Adlercreutz et al., 1995). However, after binding to estrogen receptors, those isoflavones are much less effective than estradiol and DES in translocating the binding complex to the nucleus and in stimulating the synthesis of more receptors (Folman and Pope, 1969; Martin et al., 1978), a property that is classified as antiestrogenic effect.

Isoflavones may also affect the action of estrogens indirectly by increasing the synthesis of sex-hormone binding globulin (SHBG) in the liver which elevates the level of SHBG in plasma and decreases the levels of free (non-protein bound) estrogens and testosterone in the plasma (Adlercreutz et al., 1987). Genistein also increases SHBG synthesis in human hepatocarcinoma cells (Hep-G_2) in a dose dependent manner (from 5 to 40 μm; Monsavi and Adlercreutz, 1993). These results suggest that high concentrations of

diphenolic isoflavones entering portal circulation may stimulate SHBG synthesis in the liver and a higher SHBG level means a lower metabolic clearance rate and uptake of sex hormones in many tissues, which in theory, will decrease the risk of sex-hormone dependent cancers by reducing the amount of bioactive sex hormones in the plasma.

In addition to the antiestrogenic effects (competitive binding to estrogen receptor and less effective in stimulating DNA transcription) and the stimulation of SHBG synthesis, isoflavones may decrease estrogen levels by inhibition of aromatase activities, cytochrome P-450 enzymes that catalyze the conversion of androgens to estrogens (Kellis et al., 1984; Adlercreutz et al., 1993). The mechanism of the inhibition is that isoflavones competitively bind to aromatase resulting in a decreased substrate (androgen) binding affinity.

In premenopaused women, consumption of soy protein containing 45 mg isoflavones for 1 month significantly decreased follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels, and increased menstrual cycle length by one day (Cassidy et al., 1994; Cassidy and Bingham, 1995). The increased follicular phase and menstrual cycle lengths will decrease the chance of exposure to high levels of estrogenes throughout the life span. These findings suggest that isoflavones may interfere with the release of gonadotropins by binding competitively with estrogens to receptors and interrupting the normal estrogen feedback system of hypothalamus-pituitary-gonadal axis. Considering isoflavones are capable of inhibiting several key growth enzymes, such as tyrosine-specific protein kinase, DNA topoisomerase I and II, ribosomal S6 kinase, the antiestrogenic isoflavones may inhibit

cell growth and proliferation, and thus prevent the development of sex- hormone dependent cancers such as breast and colon cancers.

Bioavailability and metabolism of isoflavones

Due to the potential health protective effects of soybean isoflavones, the bioavailability and metabolism of those compounds are of great interest for this information can provide the biological potency of these compounds and provide foundation for establishing recommended consumption levels. A series of experiments were performed in our lab to investigate human bioavailability of soybean isoflavones (Xu et al., 1994; Xu et al., 1995; Xu, 1995; Tew, 1994; Tew et al., 1996). The absorption of daidzein and genistein are dose dependent (0.7, 1.3 and 2.0 mg total isoflavones/kg body weight). The plasma concentration was 4.4 ± 2.5 μM at 6.5 hr after a dose of 2 mg total isoflavones/kg body weight. Quantitatively daidzein is more bioavailable than genistein. Average 24 hr urinary recoveries of daidzein and genistein consumed were approximately 21% and 9%, respectively. Isoflavones are cleared from the body relatively quickly. Twenty-four hours after a single dose of soy food, both plasma and urine isoflavone concentrations are minimal. Neither diet selection nor types of soy food affect the bioavailability of isoflavones (Xu, 1995). In a randomized cross-over design, eight women consumed soymilk containing 0.9 mg isoflavones /kg body weight three meals a day with basic diet, timed self-selected diet or ad libitum diet. Total 48 hr urinary excretion of daidzein was 27 ± 8%, 26 ± 10%, 26 ± 9% for the three diets, respectively. Total 48 hr genistein excretion was 20 ± 7%, 18 ± 9%, and 18 ± 8% for the three diets, respectively. In a second randomized cross-over design, ten

women consumed a single meal of tofu, cooked soybean, tempeh and texturized vegetable protein containing 0.8-1.4 mg total isoflavones /kg body weight with a controlled diet. For the four soy foods, 24 hr urinary recovery of daidzein and genistein were not statistically different. Recoveries of daidzein and genistein ranged from 38% ± 18% to 51 ± 10% and 9 ± 4% to 16 ± 5%, respectively. Dietary fat content (20% or 40% calories from fat) does not affect total isoflavone absorption measured by total urinary isoflavone excretion, suggesting that those compounds are not concomitantly transported across the enterocytes membrane and absorbed with fat (Tew, 1994). However, urinary daidzein excretion 12 hr after soy consumption was decreased by high fat diet. High fiber content diet decreases the bioavailability of isoflavones. Compared with 15 g dietary fiber, a diet containing additional 25 g of wheat fiber decreased urinary genistein excretion by 20%, whereas urinary daidzein was not affected by fiber (Tew et al., 1996). Plasma daidzein and genistein concentrations did not change with dietary fiber contents at 6.5 hr post-dosing. Compared with low fiber diet, plasma genistein level is lower in the high fiber diet at 24 hr after the dose. Furthermore, isoflavone bioavailability of long term soy consumption was also tested. In a week-long feeding trial, isoflavone absorption reached a plateau. The urinary recoveries of daidzein and genistein on the last two days of the study were the same. Interestingly, in all of the studies, fecal isoflavone recoveries were very low, only about 1-2% of ingested isoflavones was recovered from the feces. Approximately 70% of the ingested isoflavones were not recovered from both urine and feces.

The bioavailability and biological effect of soybean isoflavones may to some extent depend on their absorption and metabolism by the human body. Although the aglycones are more readily absorbed (Sfakianos et al. 1997), they only constitute 2-3% of the total isoflavone in soybean (Murphy, 1982). Over 97% of the soybean isoflavones are glucosides which are more hydrophilic than the aglycones and human digestive enzymes probably lack the ability to hydrolyze the β-glucosidic bond to release aglycones (Rowland, 1991). Because the glucosides are hydrolyzed in laboratory under 1N HCL concentration and 100°C to analyze total aglycone content (Wang et al., 1990), the high acid content of stomach secretion (which normally contains 1N HCL) might hydrolyze some of the glucosides and release aglycones for direct absorption, but this remains to be proven. Isoflavone glucosides are poorly absorbed directly in the proximal small intestine. Most of the glucosides are likely to pass on to the large bowel where microflora content is high. Once glucosides reach the colon, they can be cleaved by bacterial glucosidases, releasing free aglycones to be absorbed by the colonic mucosa (Brown, 1988). The absorbed aglycones are transported to the liver via the portal vein where they are rapidly conjugated with glucuronic acid, and to a much lesser extent with sulfate by UDP-glucuronyl transferase and sulfotransferase, respectively. Then the liver conjugation products will enter the general circulation and are excreted in the urine or with the bile into the gastrointestinal tract, where the conjugates are hydrolyzed by bacterial β-glucuronidase and the released aglycone can be reabsorbed via enterohepatic circulation. Some of the isoflavones may be

further degraded by gut flora (Hill, 1988; Winter et al., 1989; Winter et al., 1991; Cheng et al., 1969).

In vitro anaerobic incubation of genistein and daizein with human gut flora showed that gut flora could rapidly metabolize them, with fecal incubation half-lives of 7.5 and 3.3 hr for daidzein and genistein, respectively (Xu et al., 1995).

It is evident that gut flora play an important role in the absorption and metabolism of isoflavones, and thus, have great impact on the bioavailability and biological potency of those compounds. The purpose of the present study was to investigate whether human gut microflora isoflavone metabolism varied among the population and the varied gut metabolism rates contributed to the observed great inter-individual variations of isoflavone bioavailability.

MATERIAL AND METHODS

Experiment one

Subjects and protocol

Twenty healthy adults, male and female, between 21 and 45 years of age, with body weight of 61.5 ± 11.4 kg and body mass index (BMI) of 22.3 ± 2.4 kg/m^2, participated in this study (Table 1). None of the subjects took any medications, including antibiotics, for three months before and during the fecal sampling period according to their answers to the queationaire (Appendix A). Most of the subjects were omnivorous except subjects #1 and 2, who were ovolacto-vegetarians. The procedures of this study were approved by the Human Subjects Committee of Iowa State University. Informed consent of subjects was obtained in writing.

At the study's onset (day 0), twenty subjects provided freshly voided fecal samples. The fecal samples were collected in a sterile container (General Medical Corp., Des Moines, IA) and subjects were told to squeeze air out of the container. The samples were kept at room temperature and transferred to the lab within 30 min. Five subjects (Subjects #1, 2, 7, 25, and 26) provided additional fecal samples four more times during the following 10 months (on days 120, 210, 240, and 300). Subjects #6, 12, 16 and 27 provided additional fecal samples on days 240 and 300. Subjects #8, 9, 14, and 19 provided one additional fecal sample on day 300.

Chemicals

Isoflavones, daidzein (4',7-dihydroxyisoflavone) and genistein (4',5,7-trihydroxyisoflavone), were synthesized and generously provided by Dr. Murphy's lab at Iowa State University (Song and Murphy, 1997). Brain-heart infusion (BHI) dehydrated media was purchased from DIFCO Laboratories (Detroit, MI). Cysteine hydrochloride and resazurine were

Table 1. Subject information for experiment one[1]

Subject #	Gender	Ethnicity[2]	Age	Weight (kg)	Body mass index (kg/m^2)	Bowel movement[3]
1	M	C	33	43.8	18.7	R
2	F	C	32	52.6	20.0	R
6	F	As	38	40.8	17.6	R
7	M	As	33	77.0	26.6	RR
8	F	C	45	60.0	23.4	R
9	M	As	25	72.5	22.4	R
10	M	C	31	55.0	21.0	RR
11	M	C	36	85.0	23.5	R
12	F	As	31	58.9	24.5	II
13	M	C	36	70.0	24.2	R
14	F	C	40	61.2	22.5	R
16	F	AA	21	68.0	25.6	I
18	F	As	29	56.0	19.8	R
19	M	C	34	70.2	22.2	R
20	F	C	22	52.3	19.7	I
22	F	As	21	45.0	20.0	R
23	F	C	32	60.3	22.1	I
25	F	As	35	62.9	23.1	R
26	M	As	35	67.8	24.0	R
27	M	As	33	70.2	24.2	II
$\bar{x} \pm SD$			30.7 ± 9.0	61.5 ± 11.4	22.3 ± 2.4	

[1]All of the subjects were ominivorous except Subjects #1 and #2 who were ovolacto-vegetarians.

[2]Ethnicity: As = Asician, AA= Arican American, C = Caucasian.

[3]R = regular movement (once /day), RR = frequent movement (more than once/day), I = irregular movement (once/2 days), II = slow movement (one or twice/week).

purchased from Aldrich Chemical Company (Milwaukee, WI). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used. Other HPLC solvents were from Fisher Scientific Co. (Pittsburgh, PA).

Anaerobic incubation

Brain-heart infusion culture medium was made according to the manufacture's instructions. Cysteine hydrochloride (0.5 mg) and resazurine (1 mg) were added to 1 L of culture medium to serve as reducing agent and O_2 indicator, respectively. The culture medium was adjusted to pH 7.0 and autoclaved for 20 min under anaerobic conditions. In brief, brain-heart infusion medium was placed in a 1L Erlenmyer flask containing 1 mg of resazurine and heated to boiling to liberate the air that dissolved in the medium. While the medium was boiled, the upper space above the medium was filled with CO_2 gas to isolate the medium from air. Cysteine hydrochloride (0.5 mg) was added to the boiling medium when the medium became colorless. Then, the flask was stopped with a cotton ball and the medium was autoclaved at 121 °C for 20 min. Daidzein and genistein were dissolved in culture medium at concentrations of 590 μmol/L, respectively. This standard solution was autoclaved under the same condition as the culture medium. Freshly voided fecal samples were diluted tenfold with sterilized brain-heart infusion culture medium and homogenized for 10 min with a Stomacher Lab-Blender 400 (Tekmar® company, Cincinnati, OH) under anaerobic conditions. The homogenate was immediately transferred to 50 mL sterile centrifuge tubes filled with N_2 gas and centrifuged at 3000 x g for 5 min at ambient temperature. Forty mL of the supernatant was quickly added to a sterilized culture tube containing 10 mL of sterilized 590 μmol/L of both genistein and daidzein. The tubes were

filled with N_2 gas and incubated at 37 °C in a Gas-Pak® anaerobic incubation jar (Fisher Scientific, Pittsburgh, PA) filled with N_2. At 0, 6, 12, 24, and 48 h intervals, 4 mL of culture mixture was taken out for analysis of isoflavone content. Tubes containing 4 mL of sterile brain-heart infusion medium and 1 mL of isoflavone standards were incubated as negative controls. Tubes containing only the supernatant without isoflavone standards were incubated as blanks. Five mL of isoflavone containing culture mixture was sterilized at 121 °C for 20 min before measurement of recovery. Negative controls and blank samples were incubated for all of the same time intervals as were fecal samples.

Sample preparation

Sep-Pak C_{18} cartridges (Waters Inc. Rainin , Woburn, MA) were activated by 5 mL of pure methanol followed by 5 mL of distilled and deionized water (DD H_2O). Two mL of culture medium was slowly loaded on the pre-activated Sep-Pak C_{18} cartridge and washed twice with 2 mL of DD H_2O. The isoflavones were eluted by 2 mL of 80% methanol in water.

HPLC analysis

The fecal isoflavones were separated by a Waters μ-Bondapak C_{18} reverse phase column (3.9 mm i.d. x 30 cm length) with gradient elution at ambient temperature as described by Murphy (1981). Two mobile phases, Milli-Q water and methanol, were supplied by two Beckman 110 solvent delivery pumps (Fullerton, CA). A Beckman model 420 microprocessor solvent flow controller was programmed to form a gradient of 40% to 65% methanol over 30 min at a flow rate of 1 mL/min. An autosampler model 708 (Allcott, St. Louis, MO) was programmed to inject 100 μl samples onto the column. Peaks were detected by a Beckman 163 variable

wavelength detector (Fullerton, CA) set at 254 nm and 0.2 AUFS sensitivity. Peak area was calculated by a model 427 integrator (Beckman, Fullerton, CA) and used to quantify isoflavones. A series of isoflavone standards (120, 60, 30, 15, 7.5, 3.8, 1.9 μmol/L) were run with the samples to quantify fecal isoflavones. The detection limit for daidzein and genistein was 0.3 μmol/L. The recovery of fecal daidzein and genistein were 95 ± 4% and 92 ± 5%, respectively.

Fecal isoflavone half-life calculation

The logarithm of isoflavone concentration, C, was plotted against incubation time intervals. Zero hour isoflavone concentration was used as the initial reactant concentration, C_0. $Ln(C_0/C) = kt$, where k was the reaction constant or the plot slope, t was the reaction time. The time needed for half of the isoflavone to disappear ($t_{1/2}$) from the culture medium was derived from the equation: $t_{1/2} = ln(2)/k$, where k was the plot slope.

Statistical analysis

All samples were run in duplicates. Statistical analysis was done by using the SAS program (SAS Institute, Inc., Cary, NC) on the Iowa State system. Analysis of variance using the general linear models (GLM) were conducted, and significant differences between groups and times were determined by calculation of the least significant difference (LSD). For each analysis, a significant different was recognized if a P value of less than 0.05 and the difference between the comparisons was greater than the LSD.

Experiment two

Subjects and protocol

The subjects were eight non-vegetarian men between 19 and 34 years of age, with body weight of 74.1 ± 7.9 kg and body mass index (BMI) of 23.0 ± 1.9 kg/m^2 (Table 2).

Table 2. Subject information and experimental design for experiment two.

Subjects				day1-10 ⟶	day 11 ⟶	day 20
Subject No.	Age	Weight (kg)	Body mass index (kg/m^2)	Washout	Feeding	Fecal sampling
1	21	63.4	19.6		Soymilk[1]	
2	34	81.5	25.2		Soymilk	
3	20	83.8	25.0		Soymilk	
4	24	63.4	21.4		Soymilk	
5	28	72.5	24.8		Soymilk	
6	32	72.0	22.7		Soymilk	
7	19	74.7	22.3		Soymilk	
8	21	81.5	23.1		Soymilk	
$\bar{x} \pm$ SD	24.8 ± 5.8	74.1 ± 7.9	23.0 ± 1.9			

[1]Soymilk diet contained a total of 1.2 mg isoflavones/kg body weight, normalized to the aglycone form of each isoflavone.

The procedures of this feeding study were approved by the Human Subjects Committee of Iowa State University. Informed consent of subjects was obtained in writing. All of the subjects were in good health and did not take any medication, including antibiotics, for over six months before and during the study.

The study lasted three weeks. The first ten-day was a washout period when all the subjects were asked to avoid any foods that contained soy products. A list of food products containing soy was given to the subjects (Appendix B). On day eleven, all the subjects consumed a soymilk containing breakfast. The breakfast consisted of 8 ounces of cranberry juice and free choice of skimmed cow's milk, white bread, strawberry jam, peanut butter, orange juice and three kinds of cereals. Each subject consumed soymilk powder (Soya Powder, Div. of Morder Products Inc., Mequon WI) containing a total of 1.2 mg isoflavones per kg of body weight, normalized to the aglycone form of each isoflavone (Song and Murphy, 1997). The soymilk powder was dissolved in 8 ounces of cranberry juice to mask the flavor of soy. Subjects were instructed not to consume anything 10 h before dosing and not to consume any other soybean foods or foods containing texturized vegetable protein, hydrolyzed vegetable protein, or soy protein isolate during the entire study. A list of such food products was provided. No adverse effects such as diarrhea were reported after soymilk dosing.

Collection of biological samples

Ten mL venous blood samples were collected into vacuum tubes containing EDTA (Fisher Scientific, Chicago, IL) by a medical technologist under stringent aseptic conditions

at the metabolic unit of the Human Nutrition Center, Iowa State University. Blood samples were collected 6 h after each dosing and immediately centrifuged at 3000 x g for 25 minutes at 4 °C (Model 4D; International Equipment Co., Needham Hts., MA). Plasma was separated and stored at -20 °C before analysis. Ten days after the feeding, one freshly voided fecal sample from each subject was collected in a sterile container (General medical Corp., Des Moines, IA). The samples were transferred to the lab within 30 min.

In vitro incubation of isoflavones with human gut microflora

The fecal samples were incubated and extracted exactly as in Experiment one.

Analytical methods

Soymilk powder analysis. The concentrations of twelve isoflavone isomers (Figure 1) in soymilk powder were measured according to the method described by Wang and Murphy (1994a). Two grams of soymilk powder was extracted with 10 mL of acetonitrile and 2 mL of 0.1 N HCl for 2 h at room temperature. The extractants were filtered through Whatman No. 42 filter paper and the filtrate was taken to dryness on a rotary evaporator at ≤30 °C. The residue was dissolved in 10 mL of 80% methanol in water. Then, this 80% methanol solution was filtered through a 0.45 μm PTFE filter [poly (tetrafluorethylene), Alltech Associates Inc., Deerfield, IL] and analyzed for isoflavone content by HPLC. Because of the molecular weight differences of the isoflavone isomers, the total isoflavone content was normalized to the aglycone form of each isoflavone as follows: total daidzein = aglycone daidzein + (daidzin x 254/416) + (acetyldaidzin x 254/458) + (malonyldaidzin x 254/522); total genistein = aglycone genistein + (genistin x 270/432) + (acetylgenistin x

270/432) + (malonylgenistin x 270/518); total glycitein = aglycone glycitein + (glycitin x 284/446) + (acetylglycitin x 284/488) + (malonylglycitin x 284/532). The total isoflavone content was the sum of total daidzein, total genistein and total glycitein.

Plasma and fecal samples analysis. Plasma sample preparation followed the method described by Lundh et al. (1988). One mL of plasma sample was treated with 10 μL of β-glucuronidase/sulfatase (H_2 type, Sigma, St. Louis, MO) and incubated at 37 °C for 16 h to liberate isoflavone aglycones. After incubation, 1 mL of 10 mM sodium phosphate buffer (pH 7.0) and 10 μL of 3.99 mM THB were added to the sample solution. Then, the sample solution was passed through the Extrelut® QE column and extracted with 2 x 8 mL ethyl acetate. The eluent was collected and dried under N_2 gas. The dried residue was dissolved in 1 mL petroleum ether: benzene (1:1) and extracted with 2 mL 0.5 M ammonium hydroxide. The mixture was vortexed by a Deluxe Mixer (American Scientific Products, McGraw Park, IL) and centrifuged (centrifuge, IEC, Needhan Heights, MA) for 5 min at 3000 x g. The aqueous phase was transferred to another centrifuge tube and the organic phase was extracted with 2 mL 0.5 M ammonium hydroxide again. The two ammonium hydroxide extracts were combined and acidified with 0.1 mL concentrated glacial acetic acid. Then, the isoflavones were extracted with 2 x 2 mL ethyl acetate. The combined ethyl acetate extracts were dried under N_2 gas. The dried residue was dissolved in 200 μL of 80% methanol in water. Fecal incubation samples were treated with trichloroacetic acid (TCA) (Sigma, St. Louis, MO) and glycine buffer to precipitate gut microfloral cells and proteins. Three mL of culture mixture was mixed with 0.6 ml of 100% methanol and 0.9 mL of 0.4 M TCA buffer

containing 0.6 M glycine (pH 2.0). The mixture was vortexed for 1 min by a Deluxe Mixer and centrifuged at 3000 x g for 15 minute at 4 °C. Prior to HPLC analysis plasma samples and the supernatant of fecal samples were filtered through a 0.45 μm PTFE filter [poly(tetrafluoroethylene), Alltech Associates Inc., Deerfield, IL). The filtrate of plasma samples and fecal samples were collected into 300 μL autosampler vial insert and 2 mL autosampler vials (Alltech Associates Inc., Deerfield, IL), respectively. The HPLC analyses were carried out on a Hewlett Packard 1050 Series HPLC system. The HPLC system consisted of a photodiode array detector (PDA), a quadruplicate pumping system, an autosampler and a HP Vectra 486/66 personal computer with Chem Station^{3D} software (Hewlett Packard Company, Scientific Instruments Division, Palo Alto, CA). Isoflavones and their possible metabolites were separated and quantified on a YMC-pack ODS-AM C_{18} reverse phase column (5 μm, 25 cm x 4.6 mm i.d.) (YMC Inc., Wilmington, NC) attached to a precolumn in-line filter (0.45 μm, Alltech, Deerfield, IL).

Elution was carried out at a flow rate of 1 ml/min at ambient temperature with the following solvent system: A = 0.1% acetic acid in Milli-Q H_2O (Millipore Co., Bedford, MA), B = methanol. Following the injection of 20 μL of sample, solvent B was increased from 30% to 50% over 50 min. and then held at 50% for 5 min. Analytes were monitored with the PDA from 200 to 350 nm. Ultraviolet absorbance spectra were recorded and area responses were integrated by Chem Station^{3D} software to identify and quantify isoflavones and their metabolites, respectively.

Daidzein glucuronide (7-glucuronic acid, 4'-hydroxyisoflavone), genistein glucuronide (7-glucuronic acid, 4',5, -dihydroxyisoflavone) were enzymatically synthesized in Dr. Hendrich's lab at Iowa State University. Genistein (4',5,7-trihydroxyisoflavone), daidzein (4',7-dihydroxyisoflavone), glycetein (5-methoxy, 7,4'-dihydroxyisoflavone) and 2,4,4'-trihydroxydeoxybenzoin (THB) standards were synthesized or purified by Dr. Murphy's lab at Iowa State University (Song and Murphy, 1997). The purities were checked by chromatographic method and were greater than 95%. Equol (4',7-dihydroxyisoflavondiol), O-Desmethylangolensin (O-DMA), and dihydrodaidzein (DHD) standards were generously provided by Plantech (Reading, England). All of the standards were dissolved in 80% methanol in water and the concentrations were determined by spectrophotomic method and corrected for purity. THB (2,4,4'-trihydroxydeoxybenzoin) was used as internal standard to assure the quality of extraction. Plasma samples were also randomly spiked with isoflavones daidzein, genistein, and glycitein standards to measure recovery (0.1 μg /100 μL of each isoflavone added to each sample). The detection limit of plasma isoflavones was 0.1 μmol/L.

Statistical analysis

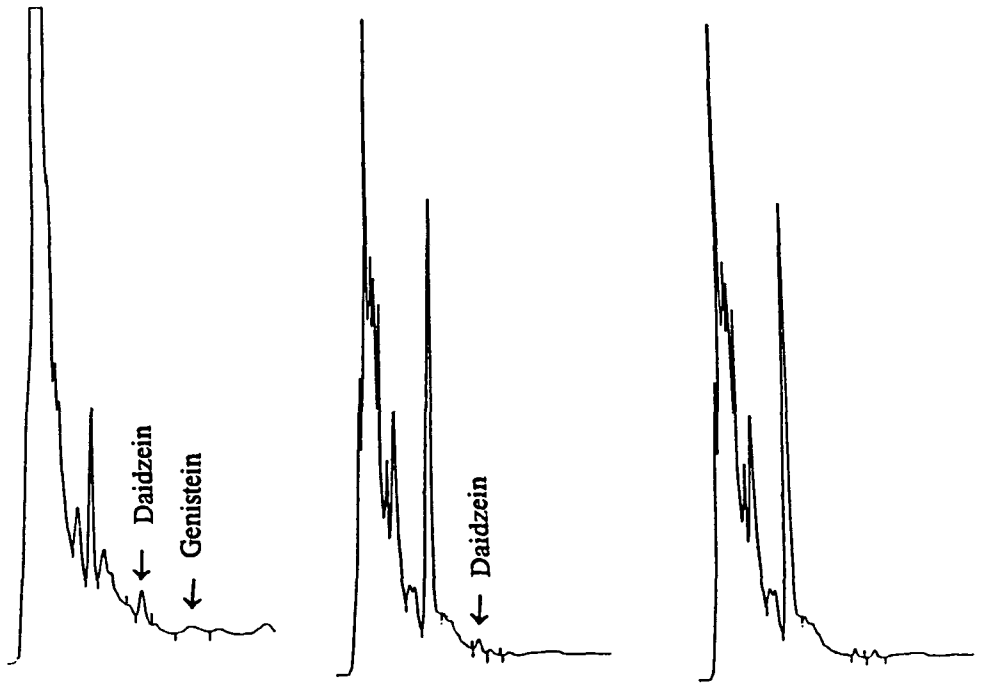
Statistical analysis was done by using Statistix, Version 4.0, developed by Analytical Software (St. Paul, MN). Spearman rank correlations method was used to analyze the data. A p value of less than 0.05 was considered to be significant.

RESULTS

Experiment one

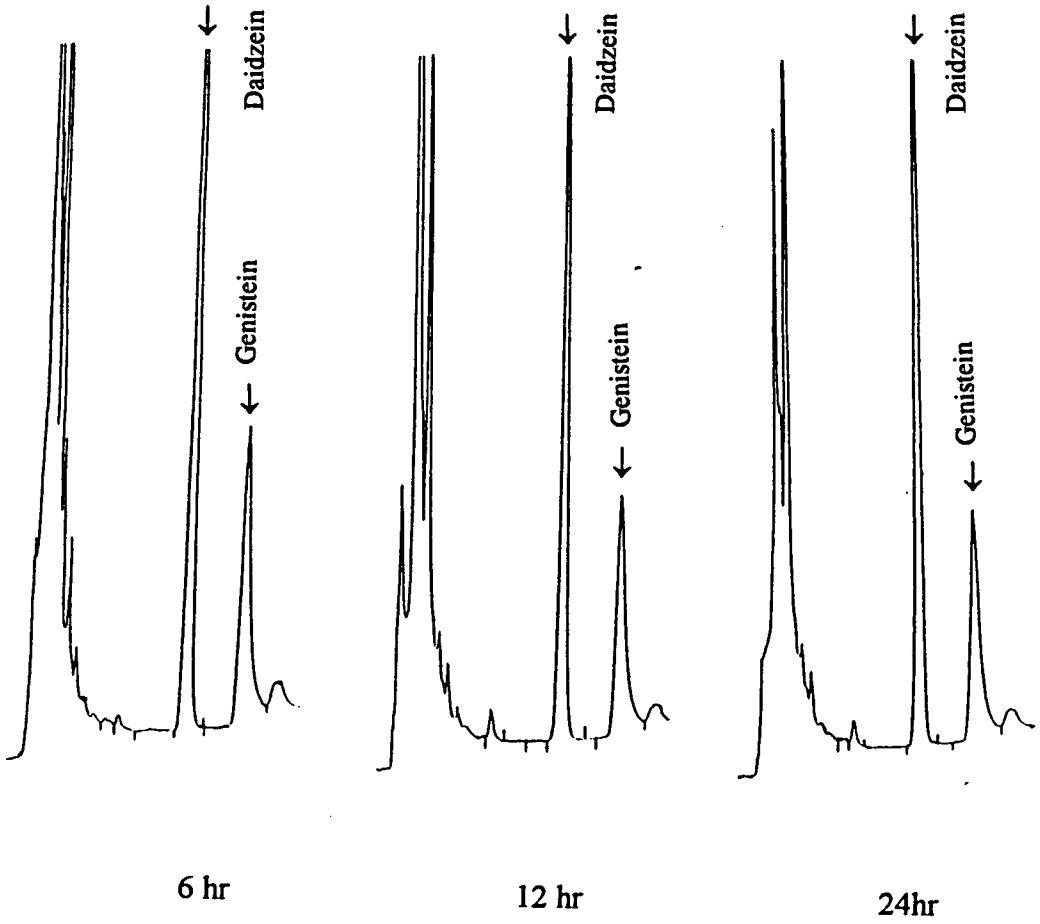
The gut microfloral isoflavone degradation rate varied considerably among the twenty subjects. As examples, chromatograms of three subjects differing markedly in their ability to metabolize isoflavones are shown in Figure 3 (A-C). The disappearance of isoflavones from the culture mixture was quite rapid for Subject #27 (Figure 3A). After 6 hr of incubation, only a minimal amount of isoflavones were detected. The fecal sample from Subject #26 showed a very slow rate of isoflavone metabolism (Figure 3B). After up to 24 hr of incubation, over 80% of isoflavones were still left in the culture media. Subject #22 showed moderate ability to metabolize the isoflavones (Figure 3C). Over 50% of daidzein and 95% of genistein disappeared after 12 hr of culture. No genistein peak was detected in the 24 hr fecal incubation sample from Subject #22.

The logarithm of isoflavone concentrations was plotted against fecal incubation time. The disappearance of isoflavones from the culture mixture followed apparent first order kinetics (Figure 4). The reaction rate constant was the slope of the regression line. The apparent first order reaction rate constant k was the negative slope of the regression line, and the isoflavone half-life ($t_{1/2}$), the time needed for half of the isoflavone to disappear from the culture media, was calculated from the equation $t_{1/2} = \ln(2)/k$. Figure 4A showed that Subject #27 had very strong ability to metabolize isoflavones, whereas the slopes of the lines describing the fecal isoflavone degradation by Subject #10 and #26 were much less than that of Subject #27 (Figure 4B and 4C).



A

Figure 3. HPLC chromatograms of fecal incubation samples from Experiment One monitored at 254 nm. A Waters μ-Bondapak C_{18} reverse phase column was used to separate daidzein and genistein and the elution was carried out at a flow rate of 1.0 mL/min with the following linear gradient: 40% methanol in H_2O to 65% methanol in H_2O over 30 min at room temperature. (A) fecal sample from Subject #27 who fits the high rate isoflavone metabolism phenotype.



B

Figure 3 (Continued) (B). Fecal sample from Subject #26 who had poor ability to metabolize isoflavones and was identified as having the low rate isoflavone metabolism phenotype.

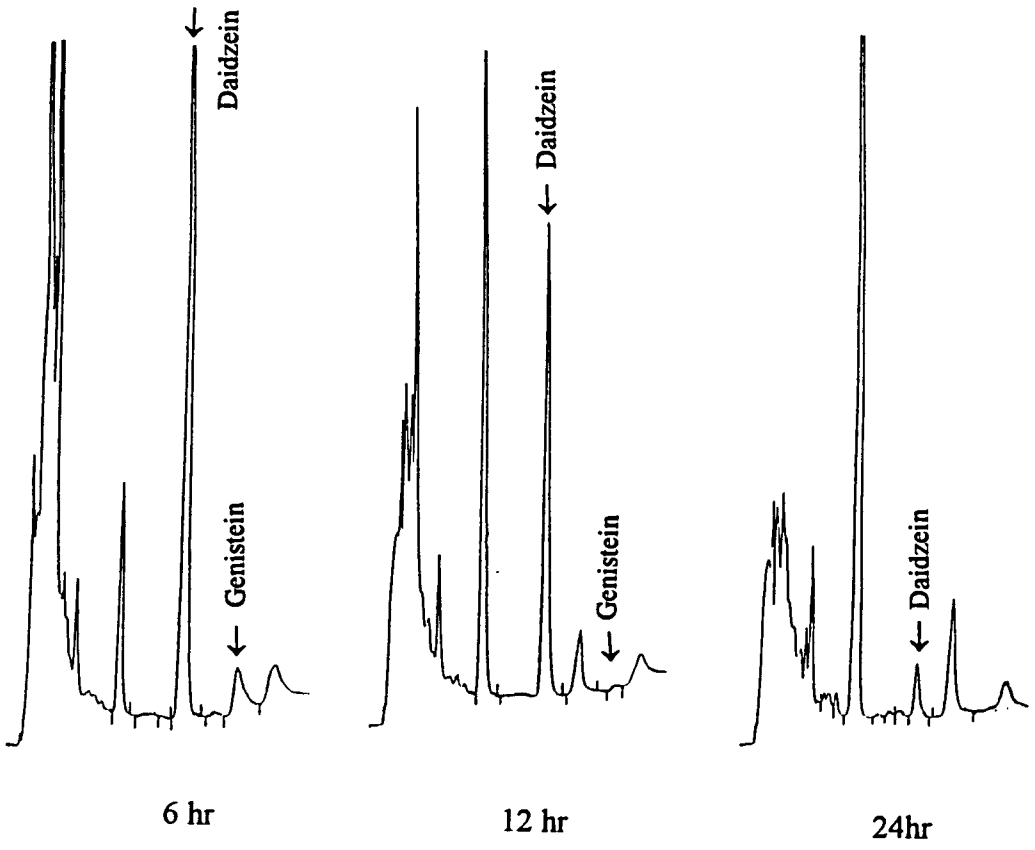


Figure 3 (Continued) (C). Fecal sample from Subject #22 who fits the moderate rate isoflavone metabolism phenotype.

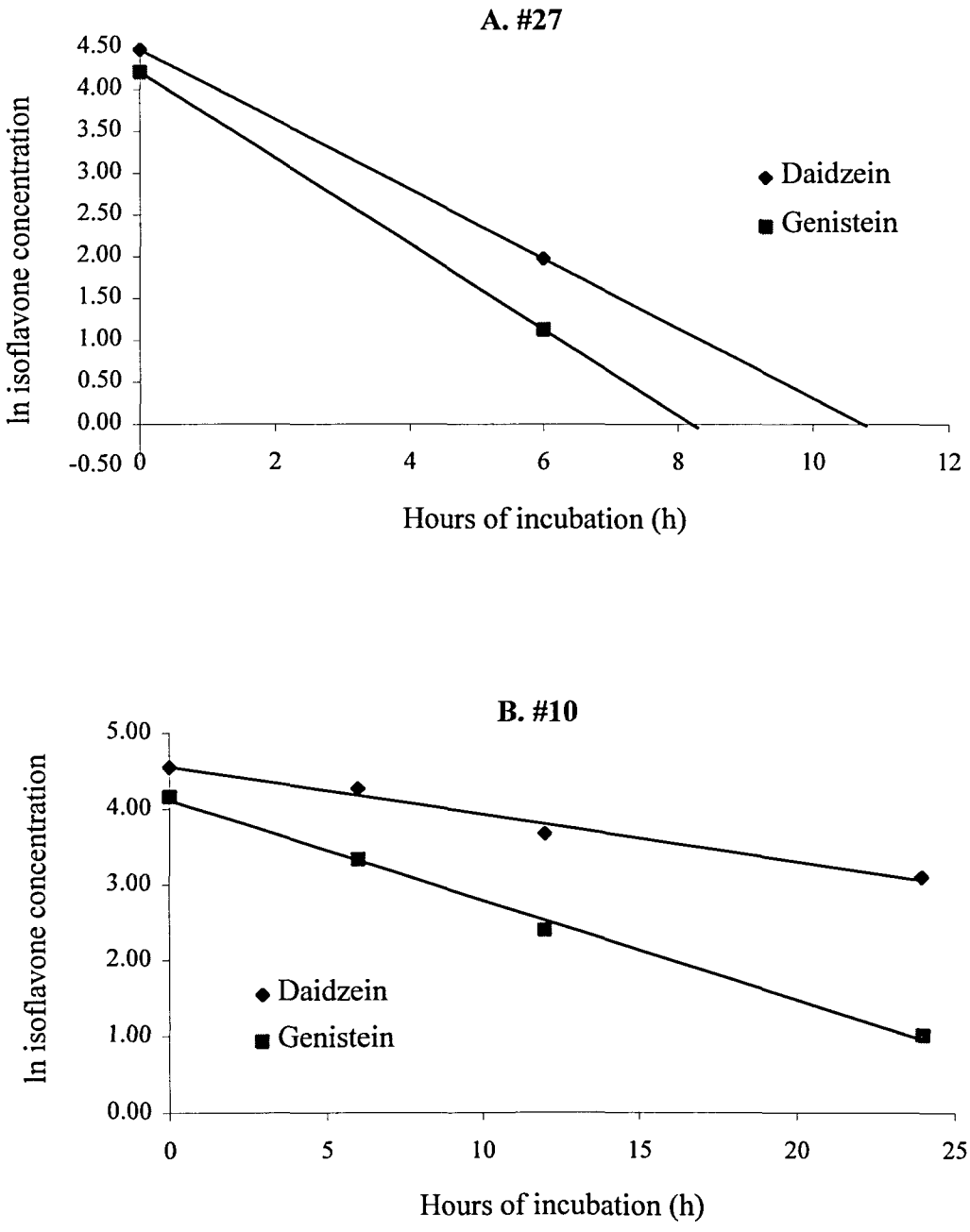


Figure 4. In vitro fecal incubation of isoflavone daidzein and genistein. Logarithm of isoflavone concentration was plotted against fecal incubation time. Negative slope of the regression line was the reaction constant k. Half-life of isoflavone = ln(2)/k. (A) Subject #27 was high rate fecal isoflavone metabolism phenotype; (B) Subject #10 was moderate rate fecal isoflavone metabolism phenotype.

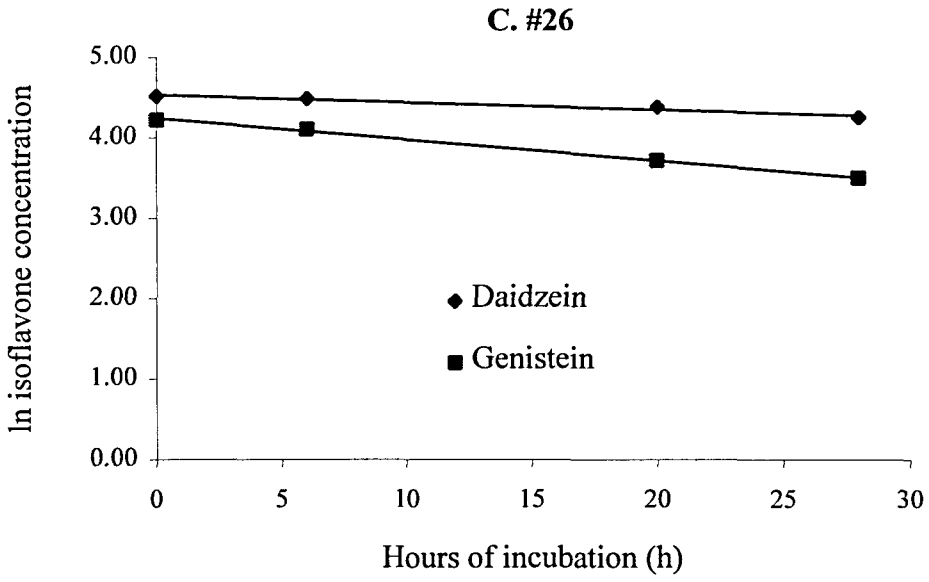


Figure 4 (continued) (C). Subject #26 was low rate fecal isoflavone metabolism phenotype.

Based on how their fecal samples could metabolize isoflavone daidzein and genistein on day 0, the twenty subjects were divided into three phenotype groups. If the sum of daidzein and genistein half-lives was less than 10 hr, the subjects (#6, 12 , 16, 23, and 27) were included into the high rate microfloral isoflavone metabolism phenotype group. If the sum of daidzein and genistein half-lives was less than 40 hr and/or the ratio of daidzein $t_{1/2}$/genistein $t_{1/2}$ was equal or greater than 2, the subjects were identified as moderate rate phenotype. The moderate group included Subjects #8, 9, 10, 11, 13, 14, 18, 19, 20, and 22. If the sum of daidzein and genistein half-lives was greater than 40 hr, the subjects (# 1, 2, 7, 25, and 26) were identified as low rate phenotype.

The fecal isoflavone metabolism reaction rate constants and fecal isoflavone half-lives of the twenty subjects over a 300-day period for daidzein and genistein, respectively, are shown in Tables 3 and 4. According to how rapidly one's fecal microflora could metabolize isoflavones, the twenty subjects were divided into three phenotypes. Five of them (Subjects #1, 2, 7, 25, and 26) showed very slow rate of fecal isoflavone metabolism with very small k values for both daidzein and genistein. The fecal samples from these subjects had very long isoflavone half-lives. The averages of the half-lives were 35.4 hr for daidzein and 23.4 hr for genistein over a ten-month period of study. Those five subjects were identified as low rate isoflavone metabolism phenotype. Subjects #6, 12, 16, 23, and 27 showed very rapid fecal isoflavone metabolism rates and very short fecal isoflavone half-lives. The $t_{1/2}$ of this group averaged 2.85 hr for daidzein and 2.13 hr for genistein over the ten-month study. These subjects were identified as the high rate fecal isoflavone metabolism

phenotype. The remaining ten subjects (Subjects #8, 9, 10, 11, 13, 14, 18, 19, 20, and 22) were identified as having a moderate rate of fecal isoflavone metabolism. The fecal isoflavone $t_{1/2}$ of the moderate rate group averaged 17.4 hr for daidzein and 5.13 hr for genistein.

The means of k and $t_{1/2}$ of daidzein and genistein for the three phenotypes at different time points are presented in tables 5 and 6, respectively. In the first month of the study (Day 0), the means of the k and $t_{1/2}$ of the three phenotypes were significantly different from each other for the two forms of isoflavones ($P < 0.001$). The mean reaction rate constant of the high rate isoflavone metabolism phenotype was 0.2702 for daidzein, which was over twenty-four fold higher than that of the low metabolism rate phenotype and about two-fold higher than that of the moderate rate phenotype. In turn, the average fecal daidzein half-life associated with the high rate phenotype was over twenty-fold and six-fold shorter than those of the low rate and moderate rate phenotypes, respectively. Comparing Tables 5 and 6, on day 0 genistein metabolism rates of the three phenotypes varied in a manner similar to the differences among phenotypes in daidzein metabolism. The three phenotypes significantly differed from each other with respect to fecal genistein metabolism reaction rate constant k and fecal genistein $t_{1/2}$ ($P < 0.001$) (Table 6). The average genistein fecal incubation half-lives were 32.6, 5.13, and 2.38 hr for low, moderate and high rate phenotypes, respectively.

On day 240, which was eight months after the initial study, the rate constant k for daidzein and genistein of the low rate phenotype were still significantly different from that of high rate phenotype ($P < 0.001$). Half-lives of genistein significantly differed ($P < 0.05$)

Table 3. The reaction rate constants (k, h^{-1}) and fecal incubation half-lives ($t_{1/2}$, h) of fecal microfloral daidzein metabolism over a 300-day period.[1,2]

	Day 0		Day 120		Day 210		Day 240		Day 300	
Subject #	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$
1	0.011	64.0	0.056	12.4	0.052	13.3	0.044	15.8	0.138	5.3
2	0.021	32.7	0.017	40.7	0.028	24.5	0.058	12.0	0.013	50.2
7	0.009	75.2	0.024	39.5	0.028	25.1	0.035	20.1	0.027	25.7
25	0.011	61.4	0.030	25.5	0.016	39.1	0.042	16.4	0.031	22.5
26	0.008	83.6	0.027	23.3	0.018	44.3	0.007	100.3	0.057	12.2
6	0.237	2.9					0.233	3.0	0.106	6.5
12	0.208	3.0					0.235	3.0	0.184	3.7
16	0.230	3.3					0.322	2.2	0.197	3.5
23	0.209	3.3					0.233	3.0	0.509	1.4
27	0.456	1.5					0.488	1.4	0.633	1.1
8	0.024	28.7							0.018	37.7
9	0.067	10.3							0.111	6.3
10	0.058	11.9								
11	0.027	26.1								
13	0.032	21.9								
14	0.051	13.5							0.130	5.4
18	0.034	20.5								
19	0.024	28.7							0.031	22.2
20	0.107	6.9								
22	0.130	5.4								

[1]Reaction rate constant k (h^{-1})was the -slope of the ln(concentration of daidzein) vs. fecal incubation time plot.

[2]The half-life (h) of daidzein in the culture medium $t_{1/2} = \ln(2)/k$.

Table 4. The reaction rate constants (k, h^{-1}) and fecal incubation half-lives ($t_{1/2}$, h) of fecal microfloral genistein metabolism over a 300-day period.[1,2]

	Day 0		Day 120		Day 210		Day 240		Day 300	
Subject #	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$
1	0.036	19.0	0.025	27.3	0.033	20.0	0.037	18.8	0.105	6.6
2	0.017	41.8	0.028	24.7	0.015	46.3	0.085	8.1	0.028	24.5
7	0.018	37.6	0.024	39.5	0.089	7.9	0.092	7.5	0.049	14.1
25	0.017	40.1	0.032	21.7	0.022	31.7	0.025	28.3	0.020	34.9
26	0.029	24.3	0.035	20.1	0.033	20.9	0.084	8.3	0.043	16.1
6	0.320	2.3					0.345	2.0	0.394	1.8
12	0.283	2.5					0.301	2.3	0.650	1.1
16	0.316	2.2					0.518	1.4	0.372	1.9
23	0.275	2.5					0.224	3.1	0.243	2.9
27	0.295	2.4					0.435	1.6	0.345	2.0
8	0.140	5.0							0.097	7.4
9	0.313	2.2							0.376	1.9
10	0.130	5.3								
11	0.065	10.7								
13	0.171	4.1								
14	0.088	7.9							0.229	3.0
18	0.163	4.3								
19	0.153	4.5							0.224	3.1
20	0.147	4.7								
22	0.262	2.6								

[1]reaction constant k (h^{-1}) was the -slope of the ln (concentration of genistein) vs. fecal incubation time plot.

[2]The half-life (h) of daidzein in the culture medium $t_{1/2} = \ln(2)/k$.

between the low and high rate phenotypes. Daidzein half-lives varied considerably among the five subjects of the low rate phenotype on day 240. Daidzein was barely metabolized by the fecal microflora from subject #26 while genistein was normally metabolized ($t_{1/2}$ = 8.27 hr). Therefore, although the mean daidzein $t_{1/2}$ of the low rate phenotype (32.9 hr) was fourteen-fold longer than the mean $t_{1/2}$ of the high rate phenotype (2.49 hr), they were not statistically different (P = 0.05).

On day 300, genistein metabolism rate constants of the three groups were significantly different (P < 0.005) (Table 6). The genistein $t_{1/2}$ of the low rate phenotype was significantly higher than the high and moderate rate phenotypes. But genistein metabolism rate was significantly higher than the low and moderate phenotypes (P < 0.05) (Table 5), whereas the low and moderate rate phenotypes were not different from each other (P = 0.05). The $t_{1/2}$ of the high rate phenotype (3.24 hr) was significantly lower than that of the low rate phenotype (23.2 hr), but not statistically lower than that of the moderate rate phenotype (17.9 hr) (P = 0.05). Daidzein half-lives of the low and moderate rate phenotypes were not statistically different from each other (P = 0.05).

The stability of the fecal microfloral isoflavone metabolic rate phenotype was tested. Figure 5 shows the mean k values over time for daidzein (A) and genistein (B). The ability to rapidly metabolize isoflavones of the high rate phenotype was a very stable characteristic over ten months of study (Table 5, 6). P values for both rate constant and $t_{1/2}$ greater than 0.6 for daidzein and greater than 0.3 for genistein showed that there was no significant different among the three test days (day 0, day 240, and day 300) for daidzein and genistein fecal

metabolic rates. The moderate rate phenotype did not change significantly between day 0 and day 300 for daidzein and genistein fecal metabolism (Table 5, 6) ($P = 0.58$ for daidzein fecal metabolism rate constant and $P = 0.54$ for genistein fecal metabolism rate constant). There was great interindividual variations over time with the low rate phenotype. Day 0 daidzein metabolism was slower than the other four test days. The $t_{1/2}$ on day 0 was significantly longer than the other four test days ($P = 0.08$). But the reaction rate constant k and $t_{1/2}$ were stable over six months (between days 120 and 300) ($P > 0.1$ for k) for this group of subjects. Genistein metabolism on day 240 was more rapid than the four of the low rate phenotype was not statistically different from the moderate rate phenotype ($P = 0.05$). On day 300 daidzein metabolism rate constant of the high rate phenotype was against other test days ($P = 0.07$ for $t_{1/2}$ and $P = 0.1$ for k). There were no significant difference in genistein rate constant and $t_{1/2}$ on days 0, 120, 210, and 300 for the low rate phenotype (Tables 5, 6).

The three fecal isoflavone metabolism phenotypes seemed to be associated with their bowel movement frequencies (Tables 1, 3, 4). Feces from Subject #1 who had the least bowel movement frequency metabolized isoflavones rapidly. The fecal incubation half-lives of daidzein and genistein were less than 2 hr over 10 months. Another three high rate phenotype subjects (#12, 16, and 23) also had less frequent bowel movement. In support with this correlation, the two vegetarians and the other three subjects (#1, 2, 7, 25, and 26) having a low rate phenotypic characteristic had frequent and regular bowel movements

Table 5. LSDs and means of rate constant (k, h^{-1}) and half-life ($t_{1/2}$, h) of fecal microfloral daidzein metabolism.[1,2,3,4]

Metabolism	Day 0		day 120		Day 210		Day 240		Day 300	
rate phenotypes[5]	k[6]	$t_{1/2}$[7]	k	$t_{1/2}$	k	$t_{1/2}$	k[8]	$t_{1/2}$[9]	k[10]	$t_{1/2}$[11]
Low[12]	0.012^{Aa}	63.1^{Aa}	0.031^{B}	28.3^{B}	0.028^{B}	29.3^{B}	0.037^{Ba}	32.9^{Ba}	0.053^{Ba}	23.2^{Ba}
Moderate[13]	0.055^{Ab}	17.4^{Ab}							0.073^{Aa}	17.9^{Aa}
High[14]	0.299^{Ac}	2.4^{Ac}					0.302^{Ab}	2.5^{Ab}	0.326^{Ab}	3.2^{Ab}

[1]Rate constant k and half-life ($t_{1/2}$) in a column with different superscrips (a, b, c) were significantly different at $P \leq 0.05$.
[2]Rate constant k and half-life ($t_{1/2}$) in a row with different superscrips (A, B, C) were significantly different at $P \leq 0.05$.
[3]Logarithm concentration of daidzein was plotted against incubation time. Rate constant k was the negative slope of the regression line. Half-life (hr) of daidzein was derived from $t_{1/2}=\ln(2)/k$.
[4]LSD=Least Significant Difference ($\alpha= 0.05$).
[5]Based on the rate of fecal microfloral daidzein metabolism, the twenty subjects were divided into three phenotypes, low, moderate and high rate of fecal microfloral metabolism. The three phenotypes were significantly different from each other ($P<0.001$) at day 0 for both $t_{1/2}$ and k. Low rate was signifcantly different from high rate at days 240 and 300 ($p<0.001$) for both $t_{1/2}$ and k. At day 300, low rate and moderate rate groups were not significantly different from each other($P \leq 0.05$).
[6]LSD=0.077 for low and high phenotypes; LSD =0.067 for low and moderate, high and moderate phenotypes.
[7]LSD=15.2 for low and high phenotypes; LSD =13.2 for low and moderate, high and moderate phenotypes.
[8]LSD=0.116 for low and high phenotypes.
[9]LSD=38.9 for low and high phenotypes. Due to subject #26, the time variation was very big. If omit #26, LSD=3.6.
[10]LSD=0.202 for low and high phenotypes; LSD =0.214 for low and moderate, high and moderate phenotypes.
[11]LSD=18.3 for low and high phenotypes; LSD =19.4 for low and moderate, high and moderate phenotypes.
[12]Day 0 was different from the other four days (days 120, 210, 240 and 300). The characteristic of low rate daidzein metabolism was stable for 180 days (from day 120 to day 300) ($P=0.08$ for $t_{1/2}$ and $P=0.12$ for k). LSD=0.031for k and LSD=29.6 for $t_{1/2}$.
[13]The moderate rate phenotype for daidzein metabolism was stable between day 0 and day 300 ($P=0.58$ for $t_{1/2}$ and $P=0.25$ for k). LSD=0.061 for k and LSD=12.4 for $t_{1/2}$.
[14]The high rate phenotype for daidzein metabolism was stable over 300 days of study ($P=0.60$ for $t_{1/2}$ and $P=0.69$ for k). LSD=0.151 for k and LSD=1.7 for $t_{1/2}$.

Table 6. LSDs and means of rate constant (k, h^{-1}) and half-life ($t_{1/2}$, h) of fecal microfloral genistein metabolism.[1,2,3,4]

Metabolism	Day 0		day 120		Day 210		Day 240		Day 300	
rate phenotypes[5]	k[6]	$t_{1/2}$[7]	k	$t_{1/2}$	k	$t_{1/2}$	k[8]	$t_{1/2}$[9]	k[10]	$t_{1/2}$[11]
Low[12]	0.023^{Aa}	32.6^{Aa}	0.029^{A}	26.7^{B}	0.038^{A}	25.4^{B}	0.065^{Ba}	14.2^{Ba}	0.049^{Ba}	19.2^{Ba}
Moderate[13]	0.163^{Ab}	5.1^{Ab}							0.233^{Ab}	3.9^{Ab}
High[14]	0.299^{Ac}	2.4^{Ac}					0.365^{Ab}	2.1^{Ab}	0.400^{Ac}	1.9^{Ab}

[1]Rate constant k and half-life ($t_{1/2}$) in a column with different superscrips (a, b, c) were significantly different at $P \leq 0.05$.

[2]Rate constant k and half-life ($t_{1/2}$) in a row with different superscrips (A, B, C) were significantly different at $P \leq 0.05$.

[3]Logarithm concentration of genistein was plotted against incubation time. Rate constant k was the negative slope of the regression line. Half-life (h) of genistein was derived from $t_{1/2}=\ln(2)/k$.

[4]LSD=Least Significant Difference ($\alpha= 0.05$).

[5]Based on the rate of fecal microfloral genistein metabolism, the twenty subjects were divided into three phenotypes, low, moderate and high rate of fecal microfloral metabolism. The three phenotypes were significantly different from each other ($P<0.05$) at day 0 and day 300 for both $t_{1/2}$ and k. Low rate was signifcantly different from high rate at days 240 ($p<0.05$) for both $t_{1/2}$ and k.

[6]LSD=0.076 for low and high phenotypes; LSD =0.066 for low and moderate, high and moderate phenotypes.

[7]LSD=7.3 for low and high phenotypes; LSD =11.6 for low and moderate, high and moderate phenotypes.

[8]LSD=0.123 for low and high phenotypes.

[9]LSD=9.4 for low and high phenotypes.

[10]LSD=0.154 for low and high phenotypes; LSD =0.163 for low and moderate, high and moderate phenotypes.

[11]LSD=9.7 for low and high phenotypes; LSD =9.8 for low and moderate, high and moderate phenotypes.

[12]Day 0 was different from the other four days (days 120, 210, 240 and 300) for $t_{1/2}$. Day 240 was different from the other four days (days 0, 120, 240 and 300) for k ($P=0.07$ for $t_{1/2}$ and $P=0.10$ for k). LSD=0.032 for k and LSD=13.1 for $t_{1/2}$.

[13]The moderate rate phenotype for genistein metabolism was stable between day 0 and day 300 ($P=0.54$ for $t_{1/2}$ and $P=0.22$ for k). LSD=0.122 for k and LSD=4.9 for $t_{1/2}$.

[14]The high rate phenotype for genistein metabolism was stable over 300 days of study ($P=0.30$ for $t_{1/2}$ and $P=0.35$ for k). LSD=0.205 for k and LSD=0.8 for $t_{1/2}$.

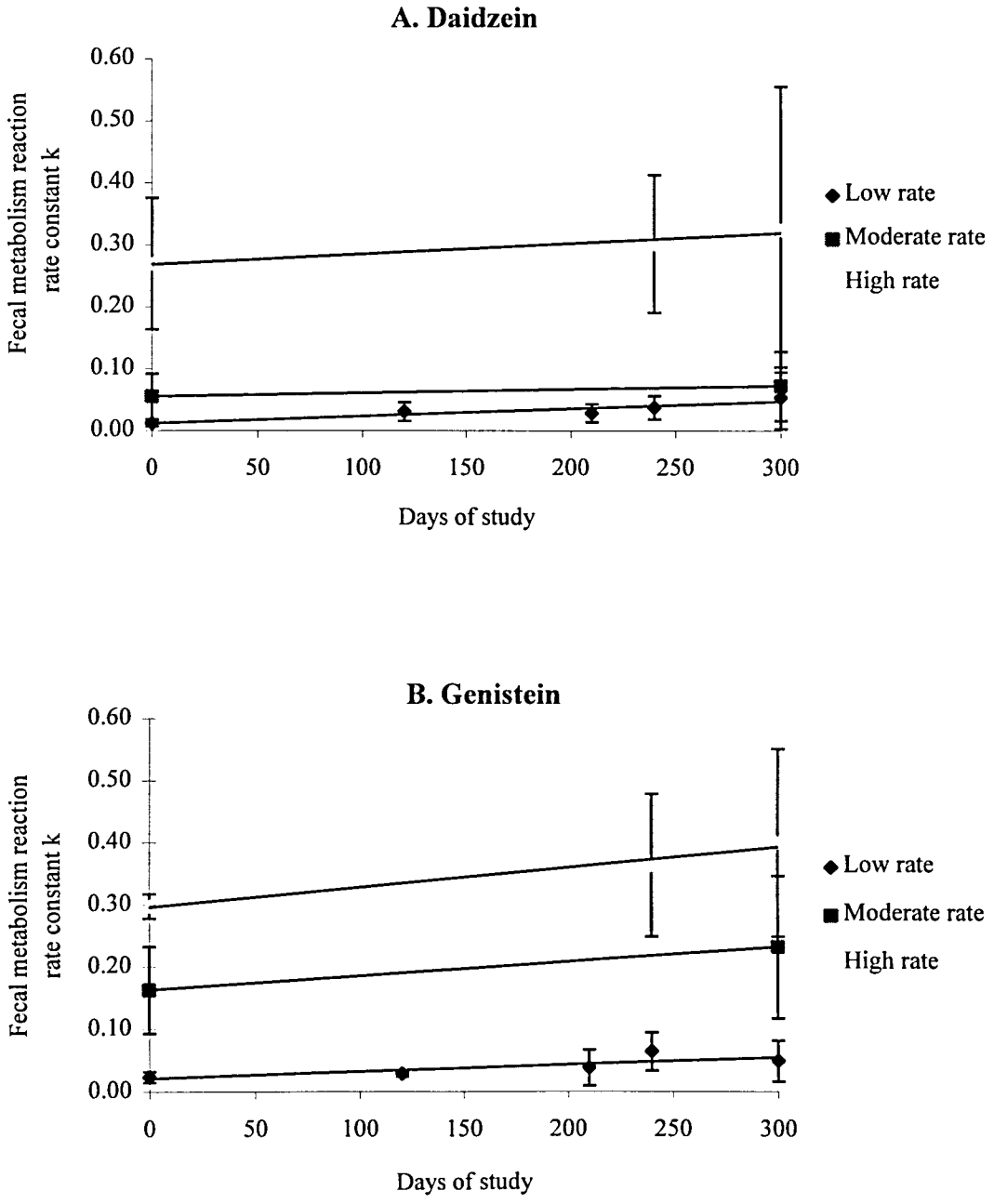


Figure 5. Fecal isoflavone metabolism reaction rate constant k vs. days of study. The three phenotypes were significantly different from each other ($P < 0.001$) at day 0 for both daidzein (A) and genistein (B).

(Table 1). Other factors, such as ethnicity, age, gender, seemed to be not associated with microfloral isoflavone metabolism rates.

Experiment two

The methanol and water mobile phases system in combination with a YMC C_{18} reverse phase column fully separated the five isoflavone isomers, three of their metabolites, and the internal standard. All the three isoflavone isomers, daidzein, genistein, and glycitein; the internal standard, THB; and the five isoflavone metabolites, daidzein glucuronide, genistein glucuronide, dihydrodaidzein, equol, and O-desmethylangolensin (O-DMA); eluted within 55 min with base line resolution. The spectrum from 210 to 350 nm of each compound was evaluated by using the photodiode array detector (PDA). Both the retention time and spectrum of each compound in samples were used for identification compared to those of the standards. The elution order and retention times (minutes) of those compounds were as follows: daidzein glucuronide, 15.04 min; genistein glucuronide, 22.60 min; dihydrodaidzein, 29.838 min; internal standard (THB), 35.85min; daidzein, 37.01 min; glycitein, 39.785 min; equol, 43.72 min; genistein, 47.02 min; O-DMA, 50.21 min. The recovery of plasma isoflavones were: daidzein, 76.5 ± 5.2%; genistein, 72.3 ± 4.8%; glycitein, 63.6 ± 4.6%; THB, 81.5± 6.4%. Recovery of the other compounds were not measured.

Soymilk powder contained 2.384 ± 0.015 mg/g total isoflavone; 42% daidzein, 50% genistein and 8% glycitein. The distribution of the 12 isflavone isomers is shown in table 7.

Table 7. Isoflavone content of the soymilk powder.

Isomers	Daidzein μg/g	Genistein μg/g	Glycitein μg/g
aglycone	53.5	58.0	14.0
glucoside	607.0	859.0	171.0
malonylglucoside	634.5	735.5	12.45
acetylglucoside	450.0	381.0	ND[2]
Total[1]	995.0	1195.0	189.0

[1]Total isoflavone was normalized to aglycone isoflavones.
[2]ND=non-detectable.

None of the plasma samples contained daidzein glucuronide and genistein glucuronide after the treatment of β-glucuronidase. This was confirmed by the HPLC retention time and spectra of purified 7-O-β-glucuronide daidzein and 7-O-β-glucuronide genistein standards. The plasma isoflavone concentrations are shown in Table 8. Plasma daidzein ranged from 0.36 to 2.87 μM and plasma genistein were from 0.65 to 4.98 μM. The plasma genistein concentration was higher than daidzein concentration 6 hours after the soymilk treatment in all of the subjects except Subject #5 (Table 8). None of the plasma samples contained detectable glycitein although about 8% of the total isoflavones in soymilk is glycitein. Dihydrodaidzein, equol and O-desmethylangolensin were not found in any of the plasma samples.

An in vitro fecal microfloral isoflavone metabolism experiment was conducted on the eight subjects. The apparent first order rate reaction rate constant k and fecal daidzein and genistein half-lives are shown in table 9. Fecal sample from Subject #1 rapidly metabolized daidzein and genistein, with $t_{1/2}$ of 1.7 hr for daidzein and 1.4 hr for genistein.

Subject #1 was identified as having the high rate phenotype. Fecal samples from Subjects #3 and #5 rapidly metabolized genistein but relatively slowly metabolized daidzein. The daidzein $t_{1/2}$ of Subjects #3 and #5 were both over 10 hr which were close to that of the low rate phenotype, whereas the genistein $t_{1/2}$ of those two subjects were close to that of the high rate phenotype. Those two subjects fit the moderate rate isoflavone metabolism phenotype. The fecal samples from the other five subjects (Subjects #2, 4, 6, 7, and 8) showed very slow reaction rates and long fecal isoflavone half-lives. These subjects fit the low rate isoflavone metabolism phenotype. Plasma daidzein concentration was negatively correlated with fecal microfloral daidzein metabolism rate constant, k_D (correlation coefficient $r = -0.74$, $P = 0.04$) and positively correlated with fecal daidzein metabolism half-life (correlation coefficient $r = 0.74$, $P = 0.04$). Plasma genistein concentration was negatively correlated with fecal microfloral genistein metabolism rate constant, k_G (correlation coefficient $r = -0.88$, $P = 0.01$) and positively correlated with fecal genistein metabolism half-life (correlation coefficient $r = 0.83$, $P = 0.01$).

Table 8. Plasma concentrations of daidzein, genistein 6 hr after the feeding.[1]

Subjects#	Daidzein μmol/L	Genistein μmol/L
1	0.36	0.65
2	2.28	3.89
3	0.59	1.26
4	2.87	4.98
5	1.04	0.82
6	1.52	1.92
7	0.95	1.43
8	1.26	3.44

[1]Glycitein was not detectable in all the plasma samples.

Table 9. The fecal microfloral metabolism rate constant k and half-lives of daidzein and genistein from the eight soymilk treated subjects.[1 2 3 4]

	Daidzein		Genistein	
Subjects	k	$t_{1/2}$ (hr)	k	$t_{1/2}$ (hr)
1	0.4164	1.7	0.5133	1.4
2	0.0279	24.9	0.0306	22.6
3	0.0624	11.1	0.0829	8.4
4	0.0336	20.6	0.0371	18.7
5	0.0299	23.2	0.1327	5.0
6	0.0760	91.2	0.256	27.1
7	0.0357	19.4	0.0458	15.1
8	0.0373	18.6	0.0396	17.5

[1]reaction constant k was the -slope of the ln (concentration of daidzein) vs. fecal incubation time plot.

[2]The half-life (hr) of daidzein in the culture media $t_{1/2} = \ln(2)/k$.

[3]Subject #1 was identified as the high rate isoflavone metabolism phenotype. Subjects #3 and 5 were the moderate rate isoflavone metabolism phenotype. The other five subjects (Subjects #2, 4, 6, 7, and 8) were the low rate isoflavone metabolism phenotype.

DISCUSSION

Our results show that human gut microflora may play important roles in isoflavone metabolism and bioavailability. Glucoside isoflavones are the predominant forms of isoflavones present in soy foods. They are apparently poorly hydrolyzed by human digestive enzymes. Therefore, after ingestion, most of the glucoside isoflavones reach the large bowel undigested and the β-glucosidic bonds are cleaved by bacterial β-glucosidase to liberate aglycones to be absorbed. The absorbed aglycones are rapidly metabolized to β-glucuronide conjugates and secreted with the bile to the alimentary tract. Gut bacteria with β-glucuronidase activity can release the aglycones by hydrolyzing the β-glucosidic bond. The released aglycones are reabsorbed and enter the enterohepatic circulation. Alternatively, those aglycones are metabolized and degraded by gut bacteria to isoflavone metabolites (such as equol and O-DMA) or some monophenolic compounds (Griffiths and Smith, 1972; Griffiths and Barrow, 1972; Winter et al., 1989), which prevent the absorption or reabsorption of isoflavones from the lower gut. Several strains of *Clostridium* and *Butyrivibrio* were reported to metabolize isoflavones and other flavonoids (Winter et al., 1989; Winter et al., 1991; Krishnamurty et al., 1970; Cheng et al., 1969). *Clostridum orbiscindens* sp. nov., *Clostridum scindens*, and *Eubacterium desmolans* isolated from human feces were found cleave the C-3 and C-4 bond on the C-ring of flavonoids quercetin to produce monophenolic fission, phloroglucinol and 3, 4-dihydroxyphenylacetic acid. The reaction could occur when quercetin either in solution or in suspension. Butyrivibrio sp. C_3 isolated from bovine rumen was capable to anaerobically ferment rutin to yield

monophenols, phloroglucinol, 3, 4-dihydroxybenzaldehyde and carbon dioxide (Krishnamurty et al., 1970). Krishnamurty and co-workers also noticed that the sugars on the ring were released by intracellular glucosidases and then the heterocyclic ring of the aglycone was cleaved. The glucosides of quercetin added to the fermentation medium were rapidly metabolized, but the aglycone quercetin was not cleaved by this strain of bacteria.

There are few bacteria in the stomach due to the high acidic content of stomach secretion. From the upper small intestine to the large bowel, the amount of bacteria increase dramatically. The aerobes are predominate in the upper portion of small intestine, whereas, anaerobes are mostly distributed in the lower part of small intestine and the large bowel (Hill, 1974). Although there are many factors that can influence gut microflora population and distribution, the cleansing action of the movement of intestinal contents down the intestine (the motility of the intestine) is probably the most important determinant of the distribution of bacteria within the intestine (Donaldson, 1968). Factors, such as genetic factors, gastrointestinal disorders, dietary factors and physical activities, that impair the normal peristalsis of the gut commonly lead to a greatly increased bacteria population in the ileum and large intestine (Hill, 1974). Our results showed that three distinguishable fecal isoflavone metabolism rate phenotypes existed in the human population. The subjects who had less frequent bowel movements tended to have high fecal isoflavone metabolism rates, e.g. fecal samples from Subjects #27 whose bowel movement were once or twice per week could rapidly metabolize daidzein and genistein with half-lives less than two hr for both daidzein and genistein. Another three high rate phenotype subjects (Subjects #12, 16, and

23) all had irregular bowel movements and their fecal incubation half-lives were all less than four hours (Tables 1, 3, and 4). On the other hand fecal samples from the two vegetarians and the other three subjects with frequent bowel movement had less ability to metabolize isoflavones. Their fecal incubation half-lives averaged over 20 hr and 14 hr for daidzein and genistein on all the five test days.

Structural diffferences of isoflavones are a determining factor of gut bacteria metabolism. Griffiths and Smith (1972) reported that isoflavones and flavonoids that possess a hydroxyl group in the 5 position of A-ring, such as genistein, but not daidzein, are much more susceptible to C-ring cleavage by rat gut bacteria. This may be the case of the moderate fecal isoflavone metabolism rate phenotype. The moderate fecal isoflavone metabolism rate phenotype was distinguished by the characteristic that their fecal bacteria could rapidly metabolize genistein, with a rate close to the rate of the high rate phenotype (half-lives were 5.13 hr on day 0 and 3.86 hr on day 300, respectively) (Table 6), but their fecal daidzein metabolism rate was similar to that of the low rate phenotype (half-lives were 17.4 hr and 17.9 hr on days 0 and 300, respectively) (Table 5).

Changes in the intestinal environment, which may be induced by gastrointestinal disease, surgery, exposure to pathogenic organisms, drugs, dietary modification, and physical activities may influence the gut microflora population and distribution. The change in gut microflora will lead to changes in microbial metabolism, which in turn may have important consequences in the bioavailability and metabolism of isoflavones. For a healthy person, dramatic dietary changes may alter the bacteria enzyme activity considerably, and

thus may change the bioavailability and metabolism of endogenous and exogenous compounds. Dietary components, resistant to digestion and absorption in the upper alimentary tract, may have great influence on the gut bacterial metabolism than other components. Tew (1996) noticed that a supplement of 25 g of wheat bran significantly decreased genistein but not daidzein bioavailability. Fiber, especially non-soluble fiber, causes fecal bulking and increases wet fecal weight due to the high water holding capacity (Cummings, 1982; Mallett et al., 1986). Furthermore, large amount of fiber in the intestine dilutes the microflora population and increases the excretion of the microflora through the feces, which may generally decrease the microbial enzyme activities. Mallett et al. (1983) reported decreased β-glucosidase and β-glucuronidase activities by dietary cellulose in rats. But certain types of fiber increased the activity of β-glucosidase and decrease the activity of β-glucuronidase (Wyatt et al., 1986). So a high fiber diet may decrease the enterohepatic reabsorption of isoflavones, thus increase fecal excretion of those compounds, by diluting the gut bacteria and decrease bacterial and β-glucuronidase activity. High fiber may also increase bacterial β-glucosidase activity which causes a high rate hydrolysis of glucoside isoflavones and thus provide more aglycones for absorption. Greater absorption of isoflavones may be responsible for the observed greater plasma isoflavone concentrations in our present study and in Xu et al. (1995) in subjects seemingly less capable of gut microflora metabolism of isoflavones. Moreover, fecal bulking caused by dietary fiber or other non-digestible dietary components may decrease the bacterial ability to metabolize and break down isoflavones giving gut mucosal cells more time to absorb aglycones and leaving more

non-metabolized isoflavones excreted through the feces. And a more frequent bowel movement may result in more fecal isoflavone excretion by reducing the bacterial fermentation time. The large individual variation in the low rate phenotype subjects over time may due to a sudden change in dietary habit and gut motility.

High meat diets, especially of beef, have been associated with increased total bacteria number, increased β-glucuronidase activity, and decreased β-glucosidase activity (Mitsuoka, 1982; Reddy et al., 1974; Goldin et al., 1978). When human subjects were shifted from a non-meat diet to a high beef diet (454 g/day), fecal β-glucuronidase increased significantly. The same phenomenon was also observed in rat studies. High beef diet also significantly increased the concentration of *Clostridia* in rats (Mitsuoka, 1982). The decreased β-glucosidase activity will decrease the hydrolysis of glucosides which limits the availability of aglycones to be absorbed. Although increased β-glucuronidase activity may liberate more isoflavone aglycones from liver conjugation products glucuronide-isoflavones, the increased concentration of gut bacteria such as *Clostridia* may rapidly metabolize and degrade those aglycones which may prevent the reabsorption of isoflavones. Furthermore, the slower bowel movement rate provides more time for bacteria to metabolize and break down isoflavones. This may explain why the high rate subject (Subject #1 in Experiment two) in the present study had low plasma isoflavone concentration and why subjects with low fecal isoflavones excretion had low plasma isoflavone concentrations (Xu et al., 1995). The fecal isoflavone metabolism of the high rate phenotype subjects varied little over time. A stable

gut environment may contribute to their stable abilities to rapidly metabolize genistein and daidzein over time.

In their bioavailability study, Xu et al. noticed that urinary and fecal isoflavone excretion varied substantially among the subjects studied after soy consumption (Xu et al. 1995). Two subjects excreted 8.6 times more daidzein and 17.5 times genistein in the feces than the other five subjects. Their urinary excretion was also significantly higher (2-3 times for daidzein and genistein). Among the two subjects excreting large amounts fecal isoflavones, plasma genistein concentration was higher than daidzein 24 hr after dosing and significantly higher than that of the five subjects excreting small amounts of fecal isoflavones although they fed a soymilk high in daidzein (56% daidzein and 44% genistein). In our present study, the plasma genistein concentration is higher than daidzein in all the subjects except one subject. The correlation between plasma genistein concentration and fecal isoflavone metabolism rate is stronger than daidzein ($r = 0.88$ for genistein at $P<0.02$ and $r = 0.74$ for daidzein at $P<0.05$).

Our present study may provide insight in the role of gut microflora on isoflavone bioavailability and biological potency. The low rate phenotype is particularly of interest for this group of people have greater ability to absorb isoflavone and easily to obtain a plasma concentration for isoflavones to exert health protective effects. On the othe hand, the high rate phenotype is of concern because their plasma isoflavone concentration generally tends to be in the low end. However, we might be able to change their gut motility and/or gut microflora content by changing their dietary habits or changing their physical activity levels

so that decrease their gut microfloral isoflavone metabolism and break down rate and thus increase their isoflavone bioavailability. Harrison et al. (1980) reported that increasing exercise (jogging for 25-45 min a day) reduced intestine transit time from > 65 hr to about 74-49 hr in subjects who had longer initial transit time, but increased intestine transit time in subjects who had shorter initial transit time. They also noticed that supplement of 30 g of wheat bran increased intestine motility. Therefore, increasing dietary insoluble fiber, such as wheat fiber, over time may be able to increase gut transit time and decrease total gut flora content and induce β-glucosidase activity which will increase the hydrolysis of glucoside isoflavones thus increasing isoflavone absorption. In addition, increasing the physical activity level of the high rate phenotype population may promote their gut motility and decrease their gut transit time and change them toward low rate phenotype.

CONCLUSION

The goal of this study was to investigate whether human gut microflora isoflavone metabolism abilities contributed to the observed great interindividual variations of isoflavone bioavailability. Our results show that gut flora play an important role in the absorption and metabolism of isoflavones. Three distinguishable phenotypes were identified according to their gut microfloral ability to metabolize isoflavones. Compared to the high fecal isoflavone metabolism rate phenotype, subjects with a low fecal isoflavone metabolism phenotype have much less microfloral ability to metabolize or break down isoflavones, thus they tend to have a high plasma isoflavone concentration after soy consumption which may more readily allow isoflavones to exert their biological effects. The moderate rate phenotype differs from the other two phenotypes. Their genistein metabolism rate which was close to the high rate phenotype was much higher than their metabolism rate of daidzein. Therefore, daidzein may be more bioavailable than genistein for this group of people. Although there is great interindividual variation, the ability of the high rate phenotype population to rapidly metabolize iosoflavones is a stable characteristic over ten months. Plasma isoflavone concentrations are correlated with fecal metabolism rate. The high rate phenotype population has relatively low plasma isoflavone concentrations.

Many factors may influence gut bacteria distribution. But for a healthy person, dietary habits and physical activities are the determinants of gut flora distribution. Therefore, we may be able to alter the dietary habit such as increasing wheat fiber intake or increase physical activities to manipulate gut physiology that favors to a decreased microfloral metabolism rate, and thus, increase isoflavone bioavailability.

APPENDIX A . QUESTIONNAIRE

This questionnaire will be kept strictly confidential.

Part I

Name _____ Phone _____

Age_____ Sex_____

Height: _____ft. _____ in. Weight _____ lbs.

Race/Ethnicity (check one):

___Caucasian, not of Hispanic origin
___African or African American, not of Hispanic origin
___ Asian or Pacific Island
___ American Indian or Alaskan native
___ Hispanic

Part II

1. Do you have any current health problems? ___Yes ___No

2. Are you currently taking any medications? ___Yes ___No
   If "Yes", please specify. _____

3. Are you taking or have you taken any antibiotics in the last 3 months? ___Yes ___No

4. Are you a vegetarian? ___Yes ___No
   If "Yes", please specify. _____

5. Are you allergic to soybeans or soy foods? ___Yes ___No

6. Do you have any food allergies other than soy (e.g., lactose intolerance)?

   ___Yes ___No
   If "Yes", please specify_____

7. How often do you consume soybean foods? (e.g. tofu, tempeh, miso, textured vegetable protein, soynuts)? Answer this question according to the "Food List".

___Never
___ A few times a week
___ Less than once a week
___ Daily

8. How frequent are your bowel movements? ___ regular ___ irregular specify.

___more than once a day
___once every day
___once every two days
___twice a week
___fewer than twice a week, specify.

9. If female, have you had ovarian disease, or loss of one or both ovaries?

___Yes ___No

10. How stressful do you find it to have your blood drawn? (check one):

___ Extremely stressful
___ stressful
___ not very stressful
___ not stressful at all

11. Will you be available between July, 1995 and July 1996? ___Yes ___No

12. The convenient time to contact you_____

Part III

1. Schedule: please mark the times you will NOT be available.

	Mon	Tue	Wed	Thr	Fri	Sta	Sun
6-7 AM							
7-8							
8-9							
9-10							
10-11							
11-12							
12-1 PM							
1-2							
2-3							
3-4							
4-5							
5-6							
6-7							

APPENDIX B. FOOD LIST

Avoid the following foods which may contain soy derivatives during the wash out weeks and the feeding period.

I. Soy Protein Isolate

Soybeans
Tofu
Soymilk, Soymilk powder
Soybean sprouts
Tempeh
miso soup
Special K frozen waffles
Carnation Instant Breakfast- chocolate malt flavored

II. Food Containing Texturized Vegetable Protein

Frozen pizza
burritos
Morningstar Farms breakfast links, patties, strips
La Choy lobster eggrolls
liquid non-diary creamers

III. Foods Containing Hydrolyzed Vegetable Protein (HVP)

most chip dips (French onion and some others)
garden vegetable flavored cheese spreads
many frozen entree's
Tombstone frozen pizza with meat
some franks (*John Morrell, Homel* Light & Lean, etc.)
many sauce mixes-gravy (usually brown), chili, etc.
Knorr soup mixes
Knorr dry sauce mixes except 'Pesto'
some jarred and canned gravies and broths (usually beef)
Wyler's bouillon (cubes and instant)
some canned soups (usually those containing vegetable & meat, like chicken and mushroom))
many *La Choy* foods as well as oriental style mixes, etc. (Containing HVP or soy sauce)
soysauce has soybeans or protein extracts from soybeans
Ramen noodles containing HVP and /or soy sauce powder
Heinz worestershire sauce (containing HVP in soy sauce)

Hiland Red Hot Riplets (most other chips and snacks were fine)
Uncle Ben's Rice mixes
some herbal magic salad dressing, *Girad's* salad dressing
most bacon flavored bits

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ACKNOWLEDGMENTS

Many people have assisted me during my study at Iowa State University. At this time, I would like to express my cordial appreciation.

First, I deeply thank my major professor Dr. Suzanne Hendrich for her guidance, support, understanding and assistance in my research. I was strongly impressed by her insight in science and enthusiastic working. I have greatly benefited from her guidance.

I am very grateful to Dr. Patricia A. Murphy for her direction, valuable assistance to my experiments. Whenever I need, she is always there to give me suggestions and generous help. I also appreciate her generosity for sharing her lab equipments.

I would like to thank Dr. Joan Cunnick for her direction and help to my research. I also thank her for willing to serve in my program of study committee.

My thanks also goes to Dr. Hendrich's group and Dr. Murphy's group members for their encouragement and help. I have a great pleasure time working with them.

Finally, I deeply thank my family for their support and understanding. Graduate is full of up and downs, my husband, Hequan Zhao, always gives me courage and support. I deeply thank my son Bo for his understanding and support. I deeply thank my parents for their encouragement and everlasting love.