PHYSIOLOGICAL AND MOLECULAR INDICATORS OF CHANGE IN THE INTESTINAL MICROFLORA OF POSTMENOPAUSAL WOMEN CONSUMING SOY AND FRUCTOOLIGOSACCHARIDES (FOS)

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

the Degree of Doctor of Philosophy in the

Graduate School of The Ohio State University

By

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The benefits of soy in postmenopausal women have been studied extensively. Dietary isoflavones are predominantly glycoside conjugates requiring enzymatic cleavage of the glucose moiety prior to absorption from the gut. Fructooligosaccharides (FOS) are preferentially metabolized by beneficial bacteria (e.g., Bifidobacteria and *Lactobacillus*), which produce these glucosidases. It has been reported that the feeding of FOS enhances excretion of isoflavones in Sprague-Dawley rats.

A prospective, randomized controlled trial was conducted, with 24 postmenopausal women being randomized to SOY + FOS or to SOY + Placebo. Isoflavone absorption, as estimated from urine isoflavone excretion, was measured via high performance liquid chromatography (HPLC). Stool enzyme assays (β-galactosidase) and molecular analyses of microbial growth (polymerase chain reaction product from stool-extracted DNA run with degradation gradient gel electrophoresis-DGGE) were performed. The absorption of the major isoflavones was moderately enhanced by FOS ingestion as determined by HPLC (Daidzein: 5-17%; Genistein: 17-34%, and Glycitein 12-20%). β-galactosidase activity increased significantly in the FOS group (P = 0.02), suggesting an increase in total bacteria. Microbial molecular results expressed as Diversity Index revealed statistically significant increases in bacterial number and density (P = 0.036) in women consuming soy and FOS. A significant
correlation existed between β-galactosidase and Diversity Index (r = 0.074). Non-supplement protein intake decreased significantly in both groups from Baseline to Treatment Periods (P = 0.005). Non-supplement caloric intake decreased as well. This indicates that nutrition counseling can enable postmenopausal women to adjust their diets so as to not to have a net increase in protein and calories when soy and FOS supplements are added.

These results suggest a trend for the modest enhancement of urine isoflavone absorption in the FOS group. The increase in the total resident bacteria, as evidenced by the statistically significant rise in the Diversity Index, the β-galactosidase stool enzyme activity, and the adjusted peak breath hydrogen underscore the magnitude of the physiological effects of the prebiotic, FOS, and soy on the microflora in postmenopausal women. This study provides a hypothetical model on which to base the design for further research to address the effects of soy and/or prebiotics on the microbiota of this population.
Dedicated to my husband
ACKNOWLEDGMENTS

I wish to thank my adviser, Anne Smith for her scientific guidance, attention to
detail, faith, and friendship. I am grateful to Mark Failla for his sharp intellect and for
pushing me beyond that which I thought I was capable of doing, to Steve Hertzler for his
patience and scientific insights and support, to Mark Morrison for the incredible
opportunity to work in his molecular microbiology lab, and to Rosita Schiller for her
academic contributions as well as her timely encouragement.

I am indebted to all of those scientists/doctoral students by whom I was mentored
in the various labs: Kelly Walsh, Zhongtang Yu, Sanjay Karnati, Sheela Premaraj, John
Sylvester, and Elizabeth Joseph. Thanks to Steve Schwartz, Qingguo Tian, Nuray Unlu,
and Torsten Bohn for their equol analyses and professionalism.

I am grateful for all the financial support received to successfully complete this
project: Ross Products Division (soy product and financial support); GTC Nutrition LLC
(FOS product support), the four scholarships/fellowships: 1) The Anna Broden
Fellowship and 2) Gladys Branegan Fellowship, both from The OSU College of Human
Ecology; 3) The Virginia Vivian Research Endowment, and 4) the Philanthropic
Educational Opportunities (PEO) $10,000 National Scholar Award. I also wish to
acknowledge the OSU General Clinical Research Center, supported by the NIH and on
whose unit I conducted my study; and to thank the nurses, Diane Habash, the Bionutrition Manager; and Raj Nagaraja, the Biostatistician; Jodi Griffith, who tirelessly helped with analyzing food records; and Dr. Paul Monk who gladly volunteered to be the clinical study physician; and, of course, to the wonderful, dedicated ladies who participated in this study. A big thank you goes to Tina Zians who tirelessly and professionally helped me to edit and format this document.

I must acknowledge the following who granted me permission to use/adapt figures or tables in this document: 1) International Life Sciences Insititute (Nutrition Reviews figures) 2) Mark Messina (Soy Connection table), 3) GTC Nutrition LLC (FOS diagram).

Lastly, I could not have accomplished this without the love, faith, and support of my one-in-a-million husband, Ed, and our very special boys, Connor and Tommy. Their humor and perspective kept me going. My amazing parents, siblings, and in-laws who all offered support, love and prayers and acted interested when hearing about my research. I also am humbled by all the encouragement from my St. Tim’s friends, (as well as their help with the boys), my OSU friends, and Ross friends.

Thank you all for believing in me.
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CHAPTER 1
INTRODUCTION

1.1 Background and Significance

As women approach mid-life, they continually seek new ways to address a variety of the accompanying health problems that include the following: 1) an increased risk and incidence of coronary heart disease (CHD); 2) peri-menopausal symptoms such as hot flashes; and, 3) decreased bone mineral density (BMD) and possible osteoporosis. Women in this century on average will live one-third to one-half of their lives after menopause, and many either cannot or will not comply with conventional therapies, such as hormone replacement. Many women have become proactive in researching their options for optimal health care, especially following the early termination of the Women’s Health Initiative trial which addressed the long-term effects of hormone replacement therapy (1, 2). The addition of soy to the diet is a modality recommended by some health care professionals to prevent disease or reduce the severity of symptoms associated with menopause such as hot flashes. Soy products are often considered safer than the majority of herbal therapies available to postmenopausal women.

Many studies have been conducted regarding the effect of soy products on the health problems of peri- and postmenopausal women. In an early meta-analysis of 38 clinical trials (with both women and men), a complete regression analysis was performed...
using different levels of soy protein in the diet (3). The model predicted that a daily intake of 25 grams of soy protein would be associated with an 8.9 mg/dL decrease in total serum cholesterol, and that a 50 gram soy protein intake would be correlated with a serum cholesterol decrease of 17.4 mg/dL.

The results of a more recent study suggest that the isoflavones in dietary soy may impart a protective role against cardiovascular disease (CVD) in postmenopausal women (4). Women with a high intake of genistein had a significantly lower body mass index (BMI), waist-circumference, and fasting insulin than those with negligible intakes. An adjusted analysis revealed that isoflavone intake was positively associated with HDL cholesterol and inversely related to a two-hour (2-h) post- challenge insulin following a 75-g oral glucose tolerance test.

The role of soy in alleviating peri-menopausal symptoms (specifically hot flashes) other than high serum lipids have historically been less clearly defined. In frequently cited studies, Washburn et al. (5) tested the daily provision of 20 grams of isolated soy protein with isoflavones to peri- and postmenopausal women in either one dose or two equal doses. Compared to placebo, the twice daily doses significantly reduced severity of hot flashes (P<0.004). In a similar study, Murkies et al. (6) found no significant differences between groups of menopausal women, consuming soy flour or wheat flour; both groups reporting a decrease in the frequency of hot flashes. However, in a later systematic review of the literature, Messina et al. found that there was a statistically significant relationship (P = .01) between initial hot flash frequency (if more than five (5)
hot flashes a day) and the treatment efficacy (soy isoflavone supplements or soy foods) in postmenopausal women (7).

The positive effect of soy isoflavones on the reduction of bone loss in several animal models has been reported (8-11). One of the first human clinical trials addressing this benefit was published in 1998 by Potter et al. (12). They hypothesized that isoflavones may also influence several biological processes, particularly, lipid and bone metabolism since these compounds have estrogenic activity. Sixty-six women were randomly assigned to one of three protein interventions. Two groups of women were provided with 40 g/d of soy protein which contained either 56 mg or 90 mg of isoflavones. The third group was given 40 g casein/d (the isoflavone-free group). The three groups consumed their randomly assigned protein mixtures in addition to a National Cholesterol Education Program (NCEP) Step I diet for a period of six months. There was an increase in high-density lipoprotein (HDL) cholesterol starting at week 6 in the group ingesting the 56 mg/d of isoflavones, but the increased HDL did not appear until week 18 in the group ingesting 90 mg of isoflavones per day ($P > 0.05$). Non-HDL cholesterol decreased at week 24 ($P < 0.05$) in both soy protein groups compared to the casein group. However, it was the ingestion of 90 mg/day of isoflavones significantly affected on bone mineral density. In addition to the delayed decrease in bone density compared with the control (casein diet) after six months, ingestion of the 90 mg/d isoflavone slightly increased bone mineral content of the lumber spine (trabecular bone) ($P < 0.05$). Thus, the moderate level of isoflavones was more effective in terms the lipid profile benefits, although the higher level of isoflavones had the positive effect on bone mineral content.
Overall, the authors concluded that although many questions about the use of soy in menopausal women needed to be answered, ingestion of isoflavone-containing soy products may provide an efficacious form of alternative therapy for the enhancement of health in postmenopausal women.

The results of this study led health care practitioners to question whether it would be beneficial to recommend the lower or the higher levels of isoflavones to postmenopausal women for maximum benefit for their cardiovascular and bone health. Many of the soy protein powders currently on the market contain approximately 55 mg of isoflavones per 20 g of protein, necessitating the consumption of closer to 40 g of protein (as in the Potter study) to achieve the bone-sparing effect of the soy. According to Dr. John Erdman, even though the women in this study were counseled to reduce their non-soy daily protein intake, many did NOT do so (13) and this could lead to weight gain or to renal implications resulting from consumption of excessive protein in predisposed populations.

In February of 2006, it was stated in an AHA (American Heart Association) Scientific Advisory analysis of 22 recent randomized clinical trials using isolated soy protein that the average effect of isolated soy on decreasing LDL cholesterol concentration was only about 3% (14). Similarly, the average decrease in LDL cholesterol reported in 19 soy isoflavone trials was negligible. The authors concede that although the beneficial cardiovascular effects attributed to soy protein and isoflavones from earlier studies could not be confirmed, “soy products should be beneficial to
cardiovascular and overall health because of their high content of polyunsaturated fats, fiber, vitamins, and minerals and low content of saturated fat” (14).

Many investigators continue to perform in vitro, animal, and clinical trials in an effort to further elucidate the potential benefits of soy and/or soy isoflavones.

The bioavailability of isoflavones is also an important consideration. The isoflavone genistin is a glycoside conjugate which requires a glycosidase for conversion to the aglycone derivative (e.g., genistin → genistein) for intestinal absorption. In 1999, it was reported that short term (7 day) feeding of fructooligosaccharides (FOS) enhanced the absorption of the isoflavones genistein and diadzein (15, 16). It was speculated that FOS was bifidogenic factor and bifidobacteria have glycosidase activity. However, the effects of FOS on isoflavone absorption in humans were unknown. Studies in humans are needed to corroborate these results. It has been recently shown in healthy humans that isoflavone glycosides (e.g., genistein and daidzein) must be converted to their aglycones in order to be absorbed (17).

If FOS enhances isoflavone absorption, consumption of less soy protein may be required to achieve the likely beneficial effects associated with these phytoestrogens. FOS is also associated with many health benefits not imparted by soy supplements alone, such as digestive tract health (18), reduced serum lipid levels (19), enhanced absorption of minerals (20), and anticarcinogenic activity (21). The study of the effects of diet in the form of prebiotics on the manipulation of the microbial community is considered critical by prominent researchers in the field (22) and has recently experienced high profile coverage at premier biomedical meetings (23).
1.2 Significance

Although literature continues to include both promising and conflicting papers regarding the benefits of soy in the postmenopausal woman in terms of cardiovascular protection, bone mineral density enhancement, and vasomotor symptoms, the consensus remains that the substitution of soy for animal proteins in the diet is advantageous. The controversy associated with the benefits of soy protein and soy isoflavones may be attributed in part to the deficiency of definitive data concerning bioavailability and metabolism of soy isoflavones. It has been established that a glycosidase is required for cleavage of the glycoside from the isoflavone for absorption and distribution to target tissues. It has been demonstrated in rats that the feeding of FOS appears to stimulate growth of bifidobacteria, and it is known that bifidobacteria produce glucosidases. A human clinical trial with postmenopausal women is warranted to determine if the provision of FOS will enhance the absorption of soy isoflavones.

1.3 Research Objective and Specific Aims:

The Research Objective of this study is to determine the effect of dietary FOS on the absorption of soy isoflavones as measured by urine isoflavones.

The primary specific aim of this study was to determine whether the short term addition of FOS (9 g/d for 3 weeks) to the diet of postmenopausal women consuming 26 g soy protein/d (80 mg isoflavones) will enhance the absorption of the isoflavones as measured by urine isoflavones.
Several response variables were explored. The secondary response variables included the following:

- To examine changes in the gut microflora via molecular techniques in patients ingesting SOY + PLACEBO and those taking SOY + FOS.

- To assess β-galactosidase activity (as an indirect measure of bacterial enzymatic activity) in the stool samples from patients ingesting SOY + PLACEBO and those taking SOY + FOS.

- To determine if a correlation exists between β-galactosidase activity and the diversity index calculated from molecular techniques involving the stool.

- To determine intestinal gas production during the treatment period by measuring breath hydrogen.

- To ascertain the subjects’ compliance (adherence) to the study intake guidelines for daily protein (soy and non-soy protein) and to determine the effect of soy protein intake on daily protein intake by comparing food records during both the baseline and treatment periods.

- To measure gastrointestinal tolerance during the baseline (SOY only) and treatment periods (SOY + PLACEBO or SOY + FOS) using a previously published Gastrointestinal Symptoms Scale.
CHAPTER 2
REVIEW OF LITERATURE

2.1 Menopause

A century ago women lived to approximately 50 years of age; thus menopause, occurring on average at age 51 years, was not an issue. Today, life expectancy for women is 80 years. Thus, women live one-third of their life in menopause (24) and quality of life during the latter years is the major impetus for clinical trials addressing menopausal health concerns.

Conjugated estrogens (e.g., Premarin) were commonly prescribed in the 1960s to treat menopausal symptoms, and by the 1980s progestin was added to prevent uterine cancer associated with estrogen only (24). During the 1990s, estrogen and progestin, referred to as “hormone replacement therapy” (HRT), were widely prescribed to prevent osteoporosis, cardiovascular disease, and vasomotor symptoms. The sudden discontinuation of the two large clinical trials addressing HRT and disease prevention (the Women’s Health Initiative - WHI, and the Heart and Estrogen/Progestin Replacement Study - HERS II studies) was unexpected by both the medical community and patients. The results of the study indicated that estrogen plus medroxyprogesterone increases (rather than decreases) the risk of myocardial infraction, stroke, venous thromboembolism, and breast cancer (1, 25-27). In 2003, it was reported that HRT is
also associated with an increased risk for dementia and urinary incontinence (28, 29).
HRT continues to be very efficacious for hot flashes, and is often used for 1-2 years around menopause to control vasomotor symptoms (30).

2.2 The Use of Phytoestrogens to Manage Menopausal Symptoms

Even prior to the cessation of the WHI and HERS II clinical trials, many women used natural products to control menopausal symptoms. At the end of 2000, however, it was documented that women spent $600 annually on these products (31). The Natural Medicine Comprehensive Database (24) lists more than 40 dietary supplements used for hot flashes and other symptoms associated with menopause. Following the early cessation of the WHI, women increasingly turned to herbal therapies, particularly phytoestrogens, in efforts to manage their menopausal symptoms (Table 2.1).

Phytoestrogens (plant estrogens) are the most popular of “natural” products used in hopes of alleviating menopausal symptoms. There are three main classes of phytoestrogens: isoflavones, lignans, and coumestans. Isoflavones are found in soy beans, chickpeas, red clover, and lentils. Lignans are present in flaxseed, with significantly lesser amounts found in lentils, whole grains, beans, fruits, and vegetables. Coumestans are present in red clover and sunflower seeds. The efficacy of soy isoflavone in vasomotor symptoms, cardiovascular disease and osteoporosis is discussed in section 2.3. A brief discussion of the efficacy of the other phytoestrogens listed in Table 2.1 ensues.
**Hormonal Medications**

Estrogens
- estrogen/progestin
- medroxyprogesterone acetate (Depo-Provera)
- megestrol acetate (Megace)

**Phytoestrogens**
- soy (Glycine max)
- red clover (Trifolium pretense)
- flaxseed (Linum usitatissimum)
- chasteberry (Vitex agnus-castus)
- kudzu (Pueraria lobata)
- alfalfa (Medicago sativa)
- hops (Humulus lupulus)
- licorice (Glycyrrhiza glabra)
- ginseng (Panax ginseng)

Table 2.1. Hormonal Medications and Sources of Phytoestrogens taken by women to ameliorate symptoms (e.g., hot flashes, night sweats) of menopause.

Red clover is widely promoted for hot flashes, but there are few studies addressing red clover for hot flashes compared to soy isoflavones. While earlier studies reported modest improvement in vasomotor symptoms (hot flash frequency) for postmenopausal women (32-34), the most recent study (the Isoflavone Clover Extract – ICE study) did not find significant improvement in frequency of hot flashes (35).

Flaxseed is not only a rich source of lignans, but also contains the omega-3 fatty acid, alpha-linolenic acid. Research suggests that the ingestion of 40 g of flaxseed/day is similar to hormone therapy in the reduction of mild menopausal symptoms (36). In another study where a wheat germ placebo was used, flaxseed improved mild hot flashes
by 35% and night sweats by 44% compared to baseline. However, such symptoms also were decreased by the same magnitude in women receiving wheat germ (37).

Although chasteberry is often recommended to relieve menopausal symptoms because components bind to estrogen receptors and stimulates progesterone synthesis, there is no reliable evidence that it attenuates menopausal symptoms (24). Kudzu, alfalfa, hops, and licorice are also promoted for menopausal symptoms but categorized under “insufficient evidence to rate” for relief of menopausal symptoms in the Natural Medicine Comprehensive Database (24). Panax ginseng is sometimes recommended for menopausal symptoms, but the estrogenic activity of ginseng is at best controversial (38, 39). On the Psychological General Well-being (PGWB) Index, a validated quality of life scale, depression in postmenopausal women was significantly improved (39). However, ginseng has been shown to induce the growth of MCF-7 breast cancer cells (38).

Soy is by far the most widely studied source of phytoestrogen, with a PubMed search yielding 7249 research and review papers for “soy” and 8602 papers for “soy isoflavone”. Following is a more in-depth discussion regarding the status of the purported beneficial health effects of soy and soy isoflavones.

2.3 Soy and Menopausal Symptoms

Soy isoflavones are diphenolic compounds possessing both estrogenic and non-estrogenic properties, qualifying them for categorization as selective estrogen receptor modulators (SERMS). It has been suggested that Asian women are less likely to experience hot flashes because of their comparatively high soy protein consumption. It is
estimated that Asian women consume 20-50 mg isoflavones in soy foods per day, while Americans consume 1 mg soy isoflavone/day (40). It is hypothesized that the low incidence of hot flashes (10%) in Asian women can be attributed to high soy consumption (41). This epidemiologic observation led to numerous clinical trials to determine the role of soy in the prevention or amelioration of the frequency and/or severity of hot flashes. The consumption of 20-60 g of soy protein (containing 34-76 mg soy isoflavones) is associated with a modest decrease in number and severity of hot flashes in some postmenopausal women (42-45). However, most agree that the heterogeneity of studies conducted to date made it difficult to definitively state that soy alleviates hot flashes (45). Messina et al. performed a systematic review of clinical trials that addressed hot flash frequency and soy foods and soy isoflavone supplements (46). They reported, based on a simple regression analysis of 13 clinical trials, that there was a significant statistical relationship between initial number of hot flashes and treatment efficacy. If a woman experienced more than five (5) hot flashes/day, hot flash frequency decreased by 5% (above placebo effects) for every initial hot flash over the five/day. It was recommended that patients with frequent hot flashes consider soy foods and isoflavone supplements for symptom alleviation.

Other studies designed with the use of concentrated soy extracts providing 35-120 mg isoflavones also report modest benefits for vasomotor symptoms (45, 47-53). One of these studies (49) was given the highest rating for study design in a 2004 review by the Agency for Health Care Research and Quality (54). In another recent study (55), conjugated estrogens were compared to a concentrated soy isoflavone extract providing
54 mg/d of genistein. The extract reduced hot flashes by 22-29% compared to 53-54% with 17 β-estradiol. A 2006 preliminary study (53) demonstrates that 60 mg of soy isoflavones twice a day (b.i.d.) is comparable to conjugated estrogens (Premarin – 0.625 mg/d) for reducing menopausal symptoms. For this effect to occur, however, isoflavone treatment required two months before subjects reported changes; whereas, the estrogen group immediately reported beneficial effects.

Not all investigators report that soy extracts significantly reduce hot flashes (56-58). This is often attributed to a high placebo response (59). An exhaustive review of all studies involving soy and menopause conducted prior to 2004 can be found in the Agency for Health Care Research and Quality document (54). In recent review of soy by Sacks et al. addressed numerous outcomes (indicators of cardiovascular disease, hot flashes, postmenopausal bone loss, and the prevention and/or treatment of various cancers) commonly addressed in the soy literature (14). Broad statements are made in this review regarding the lack of efficacy of soy in most non-cardiac outcomes (60), as well as the cardiac outcomes (see section 2.5). The proclamations regarding the non-cardiac outcomes have been criticized, for “in contrast to their (Sacks et al.) comprehensive review of cardiovascular disease, their reviews in these other areas were quite limited and cursory. More extensive reviews on these subjects with different conclusions have been published elsewhere” (46, 54, 61).

2.4 Soy and Bone Health

The plethora of evidence suggests that soy protein can improve markers of bone turnover, increase bone mineral density (BMD), or slow BMD loss (61-64). The content
of soy isoflavones in the soy protein is of importance in this context in clinical trials. At least 80-90 mg of isoflavones in 40 grams of protein seems to be required to improve BMD, and that lower amounts do not seem to be effective in most women (62-64). The exception, however, may be in Asian women. Evidence exists where postmenopausal Japanese women consuming 54 mg soy isoflavones daily seem to have higher BMD than Japanese women consuming less (65). This may be due to the increased sensitivity to the effects of soy isoflavones in Asian women (59). Studies regarding soy intake and fracture rate are few (61); but one such study in Asian women provides preliminary evidence that a higher consumption of soy foods is correlated with a lower risk of fracture development than in those consuming less soy protein, and the effect is more prominent in early postmenopause (65).

Other clinical trials have found that soy does not improve BMD (66, 67). Although the reasons for these inconsistencies are unclear, it has been proposed that this might be attributed to the variety of soy formulations (soy protein isolate vs isoflavone extracts, etc.) used, different age groups, and incongruence in study design (66, 68).

As for bone mineral content (BMC), soy may have more significant effects in women in late menopause as opposed to early menopause, women with lower body weight, and women who consume less calcium (69).

Several suggestions have been made regarding the probable mechanisms of soy in bone health. It has been proposed the weak estrogenic activity of soy is responsible for beneficial effects on bone (70). Like estrogen, soy primarily seems to affect the BMD of the lumbar spine (71). In postmenopausal women, soy increases serum osteocalcin (72),
a marker of bone formation. Genistein, has been shown to directly inhibit osteoclast activity (73), as well as promote osteoblast proliferation by inhibiting oxidative damage (74). High soy intake also seems to lower parathyroid hormone (PTH) levels, which can lead to decreased bone turnover (71).

2.5 Soy and Cardiovascular Disease

The intake of phytoestrogens (including soy isoflavones) does not seem to be correlated with a reduced risk of such primary cardiovascular events as myocardial infarction (heart attack) or cerebral vascular accident (stroke) in Western women (75).

Currently, the contribution of soy and soy isoflavones to the improvement of hyperlipidemia is being debated. An early meta-analysis (3) of the effects of soy on lipids yielded, with favorable results. Subsequent clinical trials in humans consuming soy protein also yielded results consistent with the modest improvement of hyperlipidemia (12, 42, 76-84). The Food and Drug Administration (FDA) approved a health claim for soy protein and coronary heart disease in 1999 (85). The American Heart Association (AHA) also published its first (positive for modest effects on lipid) advisory on soy protein and cardiovascular disease in 2000 (86). In the interim, some negative studies were published (14, 67, 88). In 2005, two more meta-analyses were published (54, 88) with the former reporting an approximate 3% reduction in low-density lipoproteins (LDLs), and the latter a 5.3% reduction. Sacks et al. published their updated AHA Advisory on Soy Protein, Isoflavones, and Cardiovascular Health in February 2006 (14). The current position is that soy foods (as opposed to supplements) such as tofu, soy
nuts, and soy burgers are likely to be beneficial for cardiovascular health due to their low saturated fat and high polyunsaturated fat, fiber, vitamin, and mineral content (14). Based on a meta-analysis of 22 trials, Sacks et al. estimate that soy protein directly lowers LDL by about 3%. This reduction in LDL is similar to the AHRQ analysis (54), but lower than that of Zhan and Ho (88), and all three of these meta-analyses report lower reductions than that of the early analysis by Anderson et al. (3) who reported a 12.9% decrease in LDL. The greater reduction in LDL in the meta-analysis by Anderson et al. has been attributed to the fact that many of the studies included had very hypercholesterolemic subjects (60). Triglyceride and high density lipoprotein (HDL) reductions are similar across all four meta-analyses. Table 2.2 compares the meta-analyses over the past few years in terms of soy’s contribution to lowering of blood lipids in humans.
<table>
<thead>
<tr>
<th>Reference/Year</th>
<th>LDL-related Trials Meta-analysis</th>
<th>Subjects</th>
<th>Reduction In LDL</th>
<th>Reduction In TG</th>
<th>Increase In HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anderson et al. 1995 (3)</td>
<td>31</td>
<td>564</td>
<td>12.9%</td>
<td>10.5%</td>
<td>2.4%</td>
</tr>
<tr>
<td>AHRQ 2005 (54)</td>
<td>52</td>
<td>2,000</td>
<td>3.0%</td>
<td>6.0%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Zhan &amp; Ho 2005 (90)</td>
<td>33</td>
<td>1,749</td>
<td>5.3%</td>
<td>7.0%</td>
<td>3.0%</td>
</tr>
<tr>
<td>Sacks et al. 2006 (14)</td>
<td>22</td>
<td>757</td>
<td>3.0%</td>
<td>5.0%</td>
<td>1.5%</td>
</tr>
</tbody>
</table>

Table 2.2 Cardiovascular meta-analyses and Soy Protein. Adapted from Messina et al. 2006 (Ref # 60). Used with permission.

Sacks et al. also stated that insufficient evidence exists to conclude that soy lowers blood pressure in hypertensive patients, although they did not consider a 2005 randomized controlled trial in such patients by He et al. (89) where soy protein was shown to have a significant beneficial effect on blood pressure.

Several pharmacological effects of soy have been identified that may be beneficial in cardiovascular disease (90). These include lowering of plasma homocysteine levels (91), inhibition of platelet aggregation (92), up-regulation of LDL receptors (90), and inhibition of oxidation of LDL particles (93-95). Of particular interest is that phytoestrogens, unlike estrogen, do not increase C-reactive protein (CRP) levels, an independent risk factor for coronary heart disease (96).
2.6 Soy and Cancer

Epidemiological studies suggest that Asian women who eat a high soy diet have a lower risk of breast cancer when compared with other populations (97). In Chinese women, soy intake during adolescence seems to correlate with decreased incidence of breast cancer even when these Asian women migrate to Western countries where soy is not a diet staple (98-99). This may indicate that early exposure to soy may provide protection from breast cancer later in life. It has also been suggested that the estrogenic effect of soy may be more pronounced in Asian women due to an ethnic difference (phenotype) in response (100). One recent review concludes that total dietary intake of phytoestrogens among middle-aged Western women does not seem to be positively or negatively associated with breast cancer risk (101). A 2006 meta-analysis published in 2006 with an exhaustive discussion of the issues involving soy and breast cancer included 18 epidemiologic studies published from 1978-2004. The authors conclude that, “Given many caveats and results of some experimental studies that suggest adverse effects from soy constituents, recommendations for high-dose isoflavone supplementation to prevent breast cancer or prevent its recurrence are premature” (102).

Soy has been studied in women with existing breast cancer. In a recent short-term study, women with breast cancer ingested 200 mg of soy isoflavone extract two weeks prior to the surgery, but did not appear to be any change in breast cancer cell growth (103). Longer term studies with greater numbers of breast cancer patients are required to determine the in vivo effects of soy and breast cancer.
There are epidemiologic data suggesting that increasing soy intake might lower the risk of endometrial cancer. Endometrial cancer incidence is lower in Asia where the typical diet is low in calories and high in soy, whole grains, and fruits than in Western cultures (104, 105).

In a recent case-control study of 1674 lung cancer patients and 1735 matched healthy controls, it was found that those who consume high amounts of soy isoflavones have a 44-72% lower risk of developing lung cancer compared to those who ingest small or negligible amounts. The benefit was more pronounced in men than in women (106).

At this time it is unclear why Asian men have a lower incidence of prostate cancer than men consuming the American diet. Although soy has been proposed as the agent of protection, it is difficult to differentiate whether other factors such as a lower fat intake or genetic characteristics play a role in prostate cancer protection (107-110).

The proposed mechanisms of action involving soy and cancer are multiple. Preliminary research suggests that soy isoflavones exert their anticarcinogenic effects through their weak antioxidant activity (111, 112), which lead to a decrease in lipid peroxidation (93) and oxidative DNA damage (112). In addition, soy contains other anticarcinogenic compounds such as protease inhibitors, saponins, phytosterols, and phytates (73). Early work (113) seemed to indicate that the inhibition of tyrosine kinase may also be ascribed to isoflavones, especially genistein, but direct inhibition was not shown in subsequent studies (112). Rather, in rats treated with genistein, the reduction in the reactivity of epidermal growth factor receptor (EGF-R) with antiphosphotyrosine antibodies resulted from a reduced amount of EGF-R protein (114) suggesting that
genistein’s effect was mediated through transcriptional processes as opposed to direct action on tyrosine kinase activity. Soy may also be chemoprotective for both the initiation and promotion stages of cancer because it can induce expression of the phase II enzymes, glutathione-S-transferase (GST) and quinone reductase (QR) (115). Long-term studies (in vitro, animal, clinical and epidemiological) with standardized soy preparations are needed to fully elucidate mechanisms and to assess the potential benefits and risks in the cancer process (116).

2.7 Soy and Diabetes and Diabetic Nephropathy

Clinical efficacy has been demonstrated for soy in postmenopausal women with type 2 diabetes. A soy product with 30 grams of soy protein and 132 mg of soy isoflavones was provided to postmenopausal women with type 2 diabetes for 12 weeks and lowered fasting levels of insulin, hemoglobin A1c, and LDL cholesterol (117). It has also been shown in healthy postmenopausal Chinese women that habitual soyfood consumption improves glycemic control in those with higher baseline fasting blood glucose (118). Touchi, an extract of fermented soybean has been shown in a preliminary clinical trial to act as an alpha-glucosidase inhibitor (like acarbose), and moderately lowers serum glucose, hemoglobin A1c, and triglycerides in patients with type 2 diabetes (119). Several trials have demonstrated that soy isoflavones help to prevent and treat diabetic nephropathy by reducing albuminuria (120-122).

Although it has been shown that postmenopausal women with a relatively low dietary intake of isoflavones (1.3 ± 2.4 mg/d) have a lower BMI, waist circumference,
and fasting insulin levels (4) than women who ingest no soy, the mechanism(s) responsible for benefits associated with diabetes or weight remain unknown. Preliminary evidence exists that soy might affect glycemic control by improving insulin receptor affinity, enhancing glucose transport, and increasing tissue insulin sensitivity (123).

2.8 Soy and Cognition

There is conflicting evidence as to whether soy is effective in enhancing cognitive function. File et al. (124) found that college students who ingested 100 mg isoflavones/d for 10 weeks demonstrated improved verbal and non-verbal episodic memory and mental flexibility. Another trial in premenopausal women showed that subjects given 116 mg isoflavones/d in a food matrix across two consecutive menstrual periods had improved in long-term verbal memory after in the first and last weeks of the intervention (125). Duffy et al. (126) and File et al. (127) found that 60 mg of soy isoflavones/d improved measures of cognitive function (frontal lobe function). From four published clinical randomized controlled trials and three unpublished trials, involving postmenopausal women, it appears that soy isoflavones exert a stronger effect in postmenopausal women compared to premenopausal women (125). However, another study in postmenopausal women showed no improvement in cognitive function when provided with 99 mg/d (89). The discrepancies have been attributed to different study designs and variable soy supplements and soy forms (59).
2.9 Soy and Thyroid Function

When soy first became popular for all the indications listed above, a debate ensued as to its affect on thyroid function. Soy isoflavones can block production of thyroid hormone by interfering with the thyroid peroxidase catalyzed iodination of thyroglobulin, resulting in increased thyroid stimulating hormone (TSH) and goiter (128). This however, is only of concern to women with who has inadequate iodine intake or sub-clinical hypothyroidism (129).

2.10 Soy Bioavailability and Metabolism

Defining the beneficial contributions of soy and soy isoflavones to health promotion and disease prevention is an ominous task, as discussed above. It has been stated that the elucidation of soy’s precise role may be due in part to “a lack of basic knowledge regarding their bioavailability and metabolism” (130).

Isoflavones, one of the functional ingredients in soy, are similar in structure to 17β-estradiol and bind to the estrogen receptor. Thus, isoflavones are classified as natural selective estrogen receptor modulators (SERM) (18). However, the ability of the isoflavones to bind to the estrogen β-receptor may account for only a fraction of its health promoting, since many possible beneficial mechanisms associated with soy are non-hormonal in nature and may be associated with the non-isoflavone components in soy. Many of the beneficial outcomes correlated with soy are attributed to the aglycone (without glucose) forms of the isoflavones (daidzein, genistein, and glycitein) when in fact, the β-glucoside is the naturally-occurring form in the soybean and in most soyfoods.
on the market in the Western world. Only fermented soyfoods (tofu, miso, tempeh) are naturally comprised of aglycones (131,132). Figure 2.1 displays the three glucoside and aglycone chemical structures (133).

![Isoflavone glucoside](image)

Figure 2.1 Isoflavone structure: glucosides and aglycones.
From Turner et al 2003 (Ref # 133). Used with Permission by the International Life Sciences Institute (ILSI) 2006.

As recently as 2002, it was reported that ingested isoflavone glucosides are not absorbed intact across the enterocyte. Bioavailability is contingent upon the initial hydrolysis of the glucose moiety by intestinal β-glucosidases for transport to target tissues (17). It is generally agreed that β-glucosidases are located in the intestine, although the origin of the glucosidases remains controversial. Setchell et al. (17)
substantiate the presence of intestinal (non-bacterial) β-glucosidases based on the premise that the behavior of soy isoflavones is most likely similar to that of flavonoid glucosides of quercetin in ileostomy patients. In this ileostomy study, the monoglucoside and diglucosides were efficiently hydrolyzed by β-glucosidases in the small intestine (134). This ileostomy study was consistent with earlier animal studies where the presence of both cytosolic and brush border membrane-bound β-glucosidases are described (135-138). In contrast, Turner et al. emphasize the role of bacterial glucosidases in the small intestine in addition to those in the large intestine (133). Conventional wisdom has bacterial glucosidases compartmentalized to the large intestine with the small intestine being characterized by only the cytosolic and brush border membrane-bound β-glucosidases (17, 130, 137, 139).

Regardless of origin of the β-glucosidases, it is well-accepted that after deglycosylation, the aglycone may undergo first-pass hepatic metabolism (133) or metabolism by other colonic bacteria to other isoflavone metabolites (17,133). These metabolic stages significantly affect bioavailability (glycosides are not absorbed) and bioactivity (microfloral and some of the mammalian metabolites are pharmacologically inactive (18, 133). Conjugated isoflavones can be shuttled back to the intestine via enterohepatic recycling, or they can be absorbed into general circulation (17).

Early studies demonstrate that major plasma (circulating) and urinary forms of isoflavones were glucuronides (140-143) and it was assumed that this glucuronidation (a form of conjugation with an “R” group) occurred in the liver. A study in 1997 using
everted rat intestinal sacs demonstrated that isoflavones could in fact be glucuronidated (conjugated to glucuronic acid) in the enterocyte (144), and it is now assumed that conjugation occurs in both liver and intestine (17). It is also now known that along with conjugation, the intestine has the capability to differentially shunt glucuronides across the basolateral and apical membranes of the enterocyte, with preferential transport across the basolateral membrane (17, 145). It is not known if these effluxed glucuronides use a specific transporter such as some sort of multidrug resistance-associated protein-2 (MRP2) (18). Roughly one-third of glucuronides are returned to the intestinal lumen where distal hydrolysis occurs in the colon by bacterial glucuronidases (17, 145). A relatively small proportion of isoflavones found in plasma are sulfates and conjugation is hypothesized to occur in the liver or kidneys (142).

Turner et al. (133) assert that while deglycosylation of the isoflavone glucoside can be partially attributed to non-bacterial enzymes, this action is minor compared with deglycosylation via gut microflora in both the colon and small intestine (133, 146). Over 30 years ago, Hawksworth et al (147) reported β-glucosidic enzymatic activity of enterococci, *Lactobacillus*, *Bacteroides*, and *Bifidobacteria*. All had strong β-glucosidase activity, with the enterococci displaying four times as much activity as the other three. Interestingly, the enterococci predominate in the small intestine, supporting the proposed role for deglycosylation and uptake of aglycones in this region (Figure 2.2). Turner et al. also provide comprehensive histograms regarding the log counts (CFU/mL) of these β-glucosidase-producing bacteria in all regions of the intestine, with significant numbers found in the jejunum and ileum (133).
Gut microflora play a crucial role in the metabolism of isoflavone aglycones. In addition to deglycosylation of glucoside, they convert isoflavones to genistein to \( p \)-ethyl phenol and daidzein to \( o \)-desmethylandangensin (\( o \)-DMA) and equol. \( P \)-ethyl-phenol is not estrogenic, \( o \)-DMA is mildly estrogenic, and equol is more estrogenic than daidzein and is superior to other isoflavones in its antioxidant activity (148). Equol appears to be produced by only 30-50% of adults consuming soy. This is suggested as an explanation as to why some subjects may experience beneficial effects of soy and others may not (148).

A fraction (1-25%) of dietary phytoestrogen load is excreted in the urine (133) and total fecal excretion of isoflavones is only 1-2% of that consumed (149). Therefore, greater than 75% of dietary isoflavones are most likely metabolized beyond
deglycosylation, which elucidates the role of gut microflora and explains the relatively low bioavailability of pharmacologically active dietary isoflavones (133).

Bioavailability studies of soy isoflavones have been conducted for years in human subjects with soy-based meals or supplements being fed and plasma, urine, and even fecal samples being collected to determine isoflavones and related metabolites. Most researchers report inter-individual variations that are most likely attributable to differences in gut microflora, (133, 150-154) and to some extent, food matrix (130).

2.11 Fructooligosaccharides and the Bioavailability of Soy Isoflavones

The manipulation of gut microflora may represent a method to affect the bioavailability of soy isoflavones. Prebiotics are substances that selectively promote the growth and activity of specific species of bacteria in the gut, particularly *Bifidobacterium* and *Lactobacillus* (155). Fructooligosaccharides (FOS) have prebiotic activity and are a class of oligosaccharides comprised of glucose linked to multiple fructose units; oligosaccharides have a chain length of two to four (2-4) fructose units. FOS are derived from Jerusalem artichokes, or are produced commercially from sucrose using an enzymatic process (155). FOS can also be consumed in fruits and vegetables, as the amount in Jerusalem artichokes is 4,380 mg per ½ cup, 293 mg in one Tbsp of onion powder, 236 mg in one medium ripe banana, or 12 mg in one clove of garlic (155). Because the beneficial dose of FOS is estimated to be nine grams or higher, the dietary intake is not always practical and a supplement is frequently used. FOS are not hydrolyzed by human digestive enzymes (156), the limiting factor being the β-2 linkage
between the fructose units. FOS consists of a mixture of fructose units (oligomers) known as 1-kestose, (GF₂), nystose (GF₃), and 1⁴β-fructofuranosynystose (GF₄) (157). Even though FOS are not affected by proximal digestive enzymes, the gut microfloral species, *Bifidobacterium* and *Lactobacillus* secrete β-fructosidase, which hydrolizes β-2 linkages to generate fructose for bacterial fermentation. FOS are not recovered in the feces, suggesting complete metabolism by microflora (158). Figure 2.3 shows the structure of the commercial supplement, Nutraflora® short chain FOS.

Figure 2.3 NutraFlora FOS Chemical Structure. Used with Permission, GTC Nutrition, 2006.
During fermentation of FOS, *Bifidobacterium* and *Lactobacillus* also secrete β-glucosidase to cleave the glucose moiety from the isoflavone glucoside. Colonic fermentation of FOS leads to increased fecal biomass, decreased ceco-colonic pH resulting in a nonsupportive environment for harmful bacteria and the production of short-chain fatty acids, acetate, propionate, and butyrate (159). The energy value of the fermented FOS for the host is 1.5-2.4 kcal per gram due to colonic fermentation (155). The beneficial effect of FOS on lipid metabolism has been found to be similar to that of dietary fiber (160). In addition, the preliminary evidence suggests FOS may protect against colon cancer by inhibiting tumor formation (161, 162).

FOS was first utilized in a soy isoflavone trial by Uehara et al. (15, 16) to determine its effects on the intestinal bioavailability of the isoflavones. Six-week old male Sprague-Dawley rats were infused with one intragastric dose of 8.5 mg genistein and 33 mg daidzein/kg body weight. They were fed a purified control diet (AIN-93G) or FOS diet (AIN-93G + 5% FOS). Blood samples were collected via the portal and central veins and by tail vein. (The portal – the central vein = hepatic uptake). Urine was collected during the 0-24 hour and 24-48 hour periods after isoflavone administration. The urinary excretion of isoflavonoids was significantly higher in the FOS group than in the control group. In the FOS group, both isoflavones were detected at 24 and 48 hours in samples collected from the tail vein. These observations provided the impetus to examine the effects of FOS on the absorption of soy isoflavones in humans.
CHAPTER 3
MATERIALS AND METHODS

3.1 Experimental Design

This was a prospective, placebo-controlled, single-blinded parallel clinical trial and was considered a pilot study. The implementation stage of this clinical trial consisted of three pivotal steps. These were: 1) Completion of clinical study Summary Sheets and Informed Consent for the clinical trial and subsequent approval of these documents by The Ohio State University’s Biomedical Science’s Institutional Review Board, IRB (Appendix A); 2) completion of an application and subsequent approval to conduct the trial at The Ohio State University’s General Clinical Research Center, GCRC, (Appendix B); and 3) Identification of gynecologists/women’s physicians in Columbus from whose practices subjects were recruited. Appendix C contains a cover letter used for physician recruitment.

3.2 Inclusion and Exclusion Criteria

Twenty-four (24) healthy postmenopausal women were recruited for this pilot study. Subject eligibility included the following criteria. 1) Subject had not taken any medication known to alter lipid, bone, or calcium metabolism including hormone replacement therapy over the past six (6) months. 2) Subject had not had a menstrual period within the 12 months before the study was initiated and was not older than 70
3) Subject had no history of gastrointestinal or malabsorptive disorders (lactose intolerance, Crohn’s disease, ulcerative colitis, gluten enteropathy, irritable bowel syndrome, short bowel syndrome secondary to surgery, etc.). 4) Subject had not taken soy products for three months and was not taking soy and or isoflavone supplements at study start. 5) Subject had not taken fructooligosaccharide supplements for the past three months and was not currently taking fructooligosaccharide supplements when signed on for the study. 6) Subject had not taken antibiotics or probiotics for the past three (3) months. 7) Subject was willing to be admitted to the GCRC two times, for approximately 20 hours each time over five (5) consecutive weeks to obtain the following: a) urine samples for urine isoflavones; b) breath hydrogen samples; c) fecal samples for molecular characterization of the intestinal microflora; d) four (4) blood draws over five weeks for plasma selenium; (data was to be used by another student as part of related study). 8) Subject was willing to complete food records and gastrointestinal tolerance scales for two of the five weeks of the study.

3.3 Study Visits and Treatments

3.3.1 Screening/Initiation Visit

The three visits conducted with each subject included, 1) The Screening/Initiation Visit, 2) The Baseline Visit, and 3) The Treatment Visit. The Screening/Initiation Visit was scheduled after the physician identified a subject who appeared to meet subject eligibility criteria. At this visit, subjects were further screened for eligibility, and if it was
determined that subjects met all criteria, subjects were asked to sign a Health Information
Portability and Accountability Act (HIPAA) and Informed Consent Forms, and provided
with a Clinical Trial participant packet (Appendix D), the clinical trial product, supplies
for and sample collection. The subject was also counseled with a comprehensive list
(Appendix E) to eliminate food inherently high in soy or FOS (other than the soy and
FOS intervention supplements) from their diets for the duration of the five-week study.
They also received instruction as to how to keep protein intakes at a similar level across
study periods. The dietitian’s checklist for the Screening/Initiation visit is found in
Appendix F. Once the subject signed the Informed Consent, she was randomized to
either Treatment A (Soy with Placebo) or Treatment B (Soy and FOS). The subject was
blinded as to which treatment group she was assigned. The dietitian was informed of the
randomization since she was the clinical study co-investigator who administered the in-
patient diet treatment during the Treatment visit in the GCRC.

3.3.2 Baseline and Treatment Visits

At this Screening/Initiation visit the subject was also scheduled for the Baseline
and Treatment Visits. The duration of the Baseline period was two weeks and the
duration of the Treatment period was three weeks for a total of five weeks. The subjects
were scheduled to be admitted to the GCRC two times during the five week study, during
the last week of each study period (for Baseline, this is week two of the study; for the
Treatment Period, week five of the study). They were admitted on a Wednesday evening
before 6 PM and were discharged Thursday afternoon at 2:00 PM (Admissions depicted by ▼ in figure 3.1 below):

![Baseline Period ▼ → Treatment Period ▼](Weeks 1-2) (Weeks 3-5)

Figure 3.1 Simple schematic timeline of GCRC admissions

### 3.3.3 Diet and Intervention During Baseline and Treatment Periods

During the Baseline Period, subjects consumed their normal diet with the exception of any aforementioned foods inherently high in soy or FOS. During the Treatment Period, subjects ingested the products associated with their randomization and also followed a normal diet devoid of foods inherently high in soy or FOS.

Subjects randomized to Treatment A prepared a Health Source® Soy Protein Shake (*Ross Products Division, Abbott Laboratories, Columbus, Ohio*) every morning with two scoops of powder and 8-fl oz of skim milk. Chocolate was the only available flavor at the time. They were instructed as to how to deliver the accurate scoop amount by leveling the full scoop with a knife or spatula. A 2-scoop serving provided 26 g protein and 80 mg isoflavones. Table 3.1 has the consumer label information from the Health Source® Shake. Treatment A subjects also received a *dextrose* placebo powder (*Sigma Chemical, St. Louis, MO*) to be dosed in the same manner as the fructooligosaccharides (FOS).
<table>
<thead>
<tr>
<th>Health Source® Soy Protein Shake Powder</th>
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</thead>
<tbody>
<tr>
<td>44g dry mix (2 scoops):</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Soy Protein (g)</td>
</tr>
<tr>
<td>Isoflavones (mg)</td>
</tr>
<tr>
<td>Calories</td>
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<tr>
<td>Total Fat (g)</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
</tr>
<tr>
<td>Sodium (mg)</td>
</tr>
<tr>
<td>Potassium (mg)</td>
</tr>
<tr>
<td>Total Carbohydrate (g)</td>
</tr>
<tr>
<td>Dietary Fiber (g)</td>
</tr>
<tr>
<td>Sugars (g)</td>
</tr>
</tbody>
</table>

Table 3.1 Health Source® Soy Protein Shake
2 scoops = 1 serving

Subjects randomized to Treatment B received the Health Source® Soy Protein Shake and the Nutraflora® Short Chain Fructooligosaccharide FOS Powder (P95), (GTC Nutrition LLC, Corn Products International, Inc., Golden, Colorado). The FOS powder was dosed incrementally to a total of nine (9) g/day by the fourth day of the intervention period (Baseline or Treatment), and subjects were instructed to take three (3) evenly divided doses to enhance tolerance.
During the week prior to each of the two admissions, subjects completed four days of dietary intake records (two weekdays and two weekend days) and completed Gastrointestinal Tolerance Scales on the same four days.

3.3.4 GCRC Admissions

Subjects were admitted to the GCRC by 6:00 PM of the Wednesday of Week Two (2) (for the Baseline GCRC admission) and the Wednesday of Week Five (for the Treatment GCRC admission). Subjects spent the night on Wednesday night and data collection commenced at 6:00 A.M on Thursday morning. See Tables 3.2 and 3.3 for GCRC Sample Collections and Meals for the Baseline and Treatment Periods.

BASELINE PERIOD VISIT

Subjects admitted to GCRC 6 PM Wednesday

Height and weight recorded

Fasted 6 PM Wednesday to 6 AM Thursday

Participated in one breath hydrogen collection 6 AM

Provided 24-hour urine collections 6 AM and 2 PM Thursday
  Co-investigator picked up remainder of 24-hr collection from patients’ homes

Provided small amount of stool between 6 PM and 2 PM Thursday
  Co-investigator picked up sample later if necessary from patients’ homes

Ate meal (regular diet – free of soy and FOS) breakfast and lunch Thursday

Table 3.2 Baseline Period Visit (Week 2) – Sample Collections and Meals
**TREATMENT PERIOD VISIT**

Subjects admitted to GCRC 6 PM Wednesday

Weight recorded

Fasted 6 PM Wednesday to 6 AM Thursday

Participated in breath hydrogen testing at 5:45 AM, 6 AM, 8 AM, 10 AM, 12 noon, and 2 PM

Provided 24-hour urine collections 6 AM and 2 PM Thursday
  
  Co-investigator picked up remainder of 24-hr collection from patients' homes

Provided small amount of stool between 6 PM and 2 PM Thursday
  
  Co-investigator picked up sample later if necessary from patients' homes

Administered randomized treatment (A or B) 6 AM Thursday

During breath hydrogen sampling Thursday, provided one (1) can Ensure Plus 11 AM and regular meal 2 PM

Table 3.3 Treatment Period Visit (Week 5) – Sample Collections and Meals
3.4 Methods of Subject Evaluation

Subjects were evaluated in terms of the following outcome measures:

- Urine isoflavone concentrations
- Urine equol concentrations
- Breath Hydrogen
- β-galactosidase concentrations of the fecal samples
- Microfloral characterization of fecal samples via molecular techniques
- Diet records; Gastrointestinal Tolerance Scales

All samples for each outcome measure (Table 3.4) were collected and analyzed by the dietitian/co-investigator with the exception of the equol measurements which were analyzed by the Dept. of Food Science and Technology (See section 3.6).

<table>
<thead>
<tr>
<th></th>
<th>BASELINE</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>URINE ISOFLAVONES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6A-2P</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>2P-6A</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
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<td>24</td>
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<tr>
<td>BREATH HYDROGEN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>144 24x6</td>
</tr>
<tr>
<td>FECAL SAMPLES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR-DGGE</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>β-GALACTOSIDASE</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>FOOD RECORDS</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>GASTROINTESTINAL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SYMPTOM RECORDS</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>TOTAL</td>
<td>336</td>
<td>336 672</td>
</tr>
</tbody>
</table>

Table 3.4 Number of biological samples and records collected and analyzed for the twenty-four subjects enrolled in the clinical trial
3.5 Urine Isoflavone Concentrations

3.5.1 Urine Collection

Twenty-four hour (24-h) urine collections were obtained from the subjects at two time points during their inpatient stays at the General Clinical Research Center (GCRC) during the five-week study. Subjects began collecting urine on the Thursday of each admission at 6:00 AM and continued the collection until 6:00 AM the following morning. Subjects stored urine in new 1-liter brown-tinted plastic screw-top disposable bottles for storage, after collecting the sample with a urine “hat” designed to fit the commode. To control for bacterial contamination and degradation of the specified analytes, 0.2 g of boric acid and 0.1 g sodium ascorbate (163) were added to the storage bottles. Subjects were provided with two urine bottles, one for the time frame of the 24-h urine collection when subjects were actually on the GCRC unit (the 6 AM-2 PM) and another to complete the urine collection after discharge from the GCRC (2 PM-6 AM). These bottles were labeled with the collection dates and times and the subjects’ identification code numbers. All urine bottles were kept at 4°C until the co-investigator poured aliquots from the bottles into 15-mL Falcon tubes. The co-investigator visited each subject at home the morning following GCRC discharge and picked up the second urine bottles and measured the appropriate aliquots of urine. The two 15-mL aliquots for each subject were frozen in the 15 mL Falcon tubes at -20°C until assayed.
3.5.2 Urinary Isoflavone Extraction

Extraction of urinary isoflavones was performed using modifications to the method of Haak et al (164). See Appendix G for procedure. Urine aliquots were thawed at room temperature, mixed by inverting 4 mLs from each aliquot and transferred to 50 mL centrifuge tubes, and centrifuged (Avanti J-25, Beckman Coulter Inc., Palo Alto, CA) at 8500 RPM (JA-17 rotor) for 10 minutes at 4°C. C₁₈ solid phase extraction (SPE) columns (Sep-pak, Alltech, Deerfield, Ill.) were placed on a vacuum manifold and each washed with 6 mL MeOH and 6 mL acetate buffer (pH 4.0). Five mLs were passed through 0.45 μm nylon filters placed on top of the C₁₈ SPE columns. Then 16.5 μL of internal standard (0.2) μmol 2', 4' dihydroxy-2-phenylacetophenone; (Indofine, Hillsborough, NJ) were added along with 1.5 mL acetate buffer (pH 4.0) to each column. A vacuum manifold was used to provide suction for filtering the above through the C₁₈ SPE column. The C₁₈ SPE columns were then washed with 4.0 mL acetate buffer (pH 4.0), 1 mL distilled water, and 1 mL methanol. The C₁₈ SPE columns were eluted into 11 mL glass vials with 7 mL methanol, followed by evaporation under nitrogen. Samples were resolubilized in 1 mL β-glucuronidase/sulfatase (460 μg/1 mL sodium acetate buffer, pH 5.0) and incubated overnight (> 14h) in a 37°C shaking water bath (Versa-Bath-S Fisher Scientific – Model #224) at 40 rpms. Following enzymatic incubation, samples were extracted with 3.5 mL diethyl ether (top phase) three times using glass transfer pipettes, organic phases were pooled, dried under nitrogen, and stored at -80°C until analyzed. Prior to HPLC injection, samples were resolubilized in 600 μL 80% methanol, vortexed, and filtered with a 0.2 μm syringe filter (Alltech).
3.5.3 Instrumentation

Isoflavones were separated based on the high-performance liquid chromatography (HPLC) methods of Murphy et al. (165) and Franke et al. (166) with modifications. The HPLC system consisted of a Waters 2695 separation module (Milford MA) and a Waters 2996 photodiode array detector (PDA) which were used to quantify urine isoflavones. The PDA was set to monitor a range or 210-400 nm. To achieve separation of the isoflavones, a Waters Nova-Pak C18 reversed-phase column (150 mm x 3.9 mm i.d., 4μm, 60 Å pore size) with a Nova-Pak C18 guard column was used.

3.5.4 HPLC Analysis and Identification of Isoflavonoids In Urine Samples

The mobile phase was consisted of: solvent A (1% acetic acid in water (v/v)), solvent B (100% acetonitrile), and solvent C (100% methanol) at a flow rate of 0.55mL/min. The injection volume was 10 μL, and components were eluted according to the linear solvent gradient scheme shown in Table 3.5. All the organic solvents were of HPLC grade.

The following of criteria were used for the identification of isoflavones in the urine samples: 1) UV absorption spectral profiles of pure isoflavone standards and, 2) observed retention times of these standards. For the generation of standard curves, stock solutions of three (3) pure crystalline standards (genistein, daidzein, and glycitein - Indofine, Hillsborough, NJ ) were prepared by dissolving 1 mg of each standard in 100 mL 80% methanol. Working solutions for each were prepared by the serial dilution (n=4x) of the standard stock solutions. The absolute concentration (g/mL) of the working
solutions was calculated using the Beer-Lambert-Bouguer Law \( A = \varepsilon b c; \ A = \) Absorbance; \( \varepsilon = \) molar extinction coefficient, \( b = \) path length; \( c = \) concentration) and by referring to established \( \varepsilon \) (molar extinction coefficients) and UV absorbance max \( (\lambda), \) (165). Wavelengths for maximum absorbance for daidzein, genistein, and glycitein were 249nm, 263 nm, and 256 nm, respectively. Standard curves were generated from the linear relationship between the calculated isoflavone concentrations and the generated peak areas (uV*Sec). The concentrations of the isoflavones in the analyzed urine samples were calculated using the standard curves and factors taking into account starting and resolubilization factors from the extraction protocol and total urine volumes. A sample calculation can be found in Table 3.6. Empower ProTM software was used for collection, peak integration, and organization of data.

<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>Flow</th>
<th>%A</th>
<th>%B</th>
<th>%C</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.55</td>
<td>75.0</td>
<td>12.0</td>
<td>13.0</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>0.55</td>
<td>75.0</td>
<td>12.0</td>
<td>13.0</td>
</tr>
<tr>
<td>3</td>
<td>14.00</td>
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<td>25.0</td>
<td>26.0</td>
</tr>
<tr>
<td>4</td>
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<td>10.0</td>
<td>45.0</td>
<td>45.0</td>
</tr>
<tr>
<td>5</td>
<td>19.00</td>
<td>0.55</td>
<td>10.0</td>
<td>45.0</td>
<td>45.0</td>
</tr>
<tr>
<td>6</td>
<td>20.00</td>
<td>0.55</td>
<td>75.0</td>
<td>12.0</td>
<td>13.0</td>
</tr>
<tr>
<td>7</td>
<td>25.00</td>
<td>0.55</td>
<td>75.0</td>
<td>12.0</td>
<td>13.0</td>
</tr>
</tbody>
</table>

**Table 3.5** Mobile phase linear solvent gradient for separation of urine isoflavones
A. 4.02 μg/mL  (calculated from HPLC Peak area and standard curve)

B. $4.02 \mu\text{g/mL} \div 5 \text{ mL} \times 0.6 \text{ mL} = 0.48$  (isoflavone amt ÷ starting volume x resolubilization factor = μg/mL)

C. $0.48 \times 1605 \text{ mL/24h} = 774.1 \mu\text{g/24h}$  (total μg/24 h)

Table 3.6 Sample calculations of urinary isoflavone content

3.6 Urine Equol Concentrations

Equol, a bioactive metabolite of daidzein (148) was also analyzed. Urine was collected and extracted for equol in the same manner as for the isoflavones. Analyses for the major isoflavone metabolites in urine samples were performed using HPLC-MS/MS as described above. HPLC conditions were identical as that used for urine isoflavone analysis. Mass spectrometry was performed using positive ion electrospray ionization on a triple quadrupole mass spectrometer (*Micromass Co. Ltd, UK*). Selected reaction monitoring (SRM) was used for equol ($m/z$ 243 > 133). The transition was determined by using the protonated molecule and the most abundant fragment ion determined from analysis of the respective standard. Typical conditions of the mass spectrometer were as follows: capillary voltage, 3.0 kV; cone voltage of 35 V; radio frequency lens 1, 50 V; source temperature, 120 °C; desolvation gas temperature, 500 °C at a flow of 16.3 L/min. Collision energy of 25 eV was used for all analytes. Equol was quantified using the calibration curves established from the standard.
3.7 Breath Hydrogen Sample Collection

FOS results in dose-related increases in breath hydrogen in humans (167) as a result of bacterial metabolism (167), thus interval samples of end-expiratory hydrogen were collected in accordance with standard technique (168). Prior to the study, the co-investigator provided training for GCRC nursing personnel for end-alveolar air breath hydrogen sample collection so that they could in turn teach the proper technique to study subjects. The clinical study co-investigator collected all the -15 min and 0-min samples for each subject for each visit, and nursing personnel collected all other timed samples from the subjects during the Treatment Period.

These samples were collected from the mouth of each subject in 60 mL plastic syringes fitted with a stopcock following a 12-hour overnight fast during each Period (Baseline and Treatment) in the GCRC. The collection syringes were labeled with the subjects’ code numbers and collection dates. During the Treatment Period, two baseline breath samples were collected at -15 min and 0-time before the subject was administered the challenge dose of SOY + FOS (Treatment) or SOY + DEXTROSE (Placebo). Sampling continued at two-hour intervals until 8 hours. A standard liquid supplement meal was provided at 11:00 AM, five hours into the testing. This meal (One 237 mL can of Ensure® Plus) is known to generate negligible hydrogen during digestion and metabolism. A full meal was provided after the eight-hour breath hydrogen test sample was collected at 2:00 PM.
3.7.1 Breath Hydrogen Analysis

Hydrogen concentrations were measured on a compact gas chromatograph microlyzer (*Quintron Microlyzer Model SC®, Quintron Instrument Company, Inc., Milwaukee, WI*). The Quintron was calibrated with three (3) dilutions of calibration gas (*Quintron Instrument Company*): calibration gas #1 = 100 ppm hydrogen (H₂) and 50 ppm methane (CH₄); calibration gas #2= 50 ppm H₂ and 25 ppm CH₄; and calibration gas #3 = 25 ppm H₂ and 12.5 ppm CH₄. Results for the calibration gas results were used to equilibrate the instrument to ± 2 ppm for hydrogen and methane and ± 0.10% for CO₂.

For each breath hydrogen sample, 20 cc of end alveolar air was injected into the Quintron by inserting the end of the stopcock valve into the sample flush tube on the Microlyzer. The output data provided by the Quintron was Observed Carbon Dioxide (CO₂), Raw Hydrogen, and Methane.

3.7.2 Breath Hydrogen (BH) Calculations

Raw hydrogen values were normalized by the following equation:

\[
\frac{5.26 \times \text{Raw Hydrogen}}{\text{Observed CO₂}}
\]

The 5.26% represents the percentage of CO₂ present in an optimal end-alveolar breath sample.

Baseline Adjusted Peak Hydrogen values were calculated by subtracting the baseline (-15 min or 0-time reading) reading from the “peak” hydrogen (highest breath hydrogen reading) over the eight (8) hours.
It was necessary that breath hydrogen values used in the calculations had to have corresponding CO₂ values of 5.0 ppm or above. This level of CO₂ was indicative of a successful end-alveolar breath collection. Thus, if one of the two baseline BH values had a low corresponding CO₂ for that reading, the other baseline value was used. If both baseline BH values had reliable CO₂ values, the one with corresponding CO₂ value closest to 5.25% was utilized in the equation.

3.8 Fecal Enzyme Activity Assay

3.8.1 Stool Collection and Handling

Subjects were provided with a stool kit at their Initiation Visit and given another following their Baseline visit. Stool was collected the day before admissions to the GCRC (both the Baseline and Treatment admissions). Each stool kit was comprised of sterile gloves, sterile wooden sticks, and two-15 mL Falcon tubes (Becton-Dickenson), and two (2) Specipans (Novation, Medegen Medical Products, Gallaway, TN) that fit onto the commode. The subjects were instructed to collect their stool in the Specipan, and then, wearing gloves, transfer the stool with the sterile wooden sticks into the two-15 mL Falcon tubes. The filled Falcon tubes were frozen at -20°C by the subjects and transported via cooler with ice packs and stored by the investigator at -80°C. Of the two
(2) Falcon tubes for each subject, one was marked for molecular work and the other for the β-galactosidase enzyme assay. Each subject had a Baseline sample, and each subject had a Treatment sample, for a total of 48 samples. Each sample was diluted to two separate dilutions, for a total of 96 samples. Each set of dilutions included (1) subject’s samples; (2) dilutions for the Baseline sample, and two (2) for the Treatment sample.

3.8.2 β-galactosidase Enzyme Assay

β-galactosidase activity was measured to assess microbial metabolic activity in the stool. Fecal dilutions were prepared by suspending 5 g of homogenized stool in 10 mL phosphate buffer solution (PBS); KH₂PO₄, 3.55 g Na₂HPO₄, and 9.0 g NaCl DI adjusted to pH 7.0. A standard β-galactosidase (β-gal) buffer was made by preparing .02 M Na₂HPO₄ (2.839g), .01 M MgSO₄·7H₂O (2.465 g), and .001 M Dithreitol (0.154g) (Sigma-Aldrich, St. Louis, MO).

A blank solution and five aliquots of 0.0125 M o-Nitrophenylβ-D-galactopyranoside solutions were used to generate the standard curve. Standard curves were prepared for each set of eight test samples. The solutions used to generate standard curves are listed in Table 3.7. β-galactosidase enzyme was included in the Lactose/D-Galactose Kit (Boehringer Mannheim-r-biopharm, Darmstadt, Germany).
### Table 3.7 Preparation of solutions for β-galactosidase standards

<table>
<thead>
<tr>
<th>Solution</th>
<th>Dilution</th>
<th>ONPG base (mL)</th>
<th>+ amt of DI H(_2)O Water</th>
<th>Amt of ONPG + DI H(_2)O – new vial (mL)</th>
<th>B-gal buffer (mL)</th>
<th>B-galactosidase (from enzyme kit) mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.3 DIH(_2)O only</td>
<td>3.0</td>
<td>0.03</td>
</tr>
<tr>
<td>1</td>
<td>1:16</td>
<td>0.250</td>
<td>3.75</td>
<td>0.3 of vial 1</td>
<td>3.0</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>1:8</td>
<td>0.250</td>
<td>1.75</td>
<td>0.3 of Vial 2</td>
<td>3.0</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>1:4</td>
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<td>3.00</td>
<td>0.3 of Vial 3</td>
<td>3.0</td>
<td>0.03</td>
</tr>
<tr>
<td>4</td>
<td>1:3</td>
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<td>0.03</td>
</tr>
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<td>1.00</td>
<td>0.3 of Vial 5</td>
<td>3.0</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**3.8.3 Dilutions, Sonication, and Spectrophotometer Readings**

The two dilutions for each sample prepared were a 1:1000 dilution and a 1:1500. The rationale for the second (1:1500) dilution was to accommodate any samples that may have had unusually high enzyme activity and to compare the spectrophotometer readings. The calculated enzyme activity values had to be within 10% of one another.

For each dilution, 0.1 g of homogenate was weighed and diluted to 10 g with additional β-galactosidase (β-gal) buffer. To prepare the 1:1000 dilution, 5.0 g of the sample was further diluted to 10 g. To prepare the 1:1500 dilution, 3.33 g of the initial diluted sample was further diluted with 10.0 g with β-gal buffer. Samples were sonicated (*Vibra-cell™ Ultrasonic Processor, Danbury, CT*) for one minute X five (5) cycles.

Sonicated samples were then combined with the chromic substrate for β-galactosidase.
(o-Nitrophenylβ-galactopyranoside - Sigma-Aldrich) and absorbance at 420 nm in the Spectrophotometer was recorded (Shimadzu UV160U, Kyoto, Japan) after 20 minutes at room temperature.

3.9 Molecular Characterization of Stool Samples

Stool was collected and handled as described under 3.8.1 above. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) is a cultivation-independent approach to characterize fecal microflora following dietary modification (169-170).

A recently published protocol which demonstrated improved PCR-quality community DNA extraction was used in this study. The extraction called for the repeated bead-beating plus column (RBB+C) method developed by Yu and Morrison (171). This method was comprised of three phases: 1) Cell lysis; 2) Precipitation of nucleic acids; and 3) Removal of RNA, protein, and purification of genomic DNA. The duration of each DNA extraction run (eight samples/run) was 11 hours.

3.9.1 DNA Extraction: Cell Lysis

Bead-beating was the manner in which cell lysis was achieved. Stool (0.25 g) was placed in 2 mL screw-cap microcentrifuge tubes with 0.4 g of sterile zirconia beads (0.3 g of 1mm beads and 0.1 g of 0.5 mm beads. One (1) mL of lysis buffer (4% (w/v) sodium dodecyl sulfate, 500 mM NaCl, and 50 mM EDTA) were added to each microcentrifuge tube. Microcentrifuge tubes were then placed on a Mini-Beadbeater™
(Biospec products, Bartiesville, OK) for three min at maximum speed. The samples were then placed in a 70°C water bath for 15 min, with gentle shaking by hand every five min.

Samples were centrifuged at 16,000x g at 4°C for five min and the supernatant was transferred to a 2-mL Eppendorf® tube. Three hundred (300) μL of lysis buffer was added to the precipitate and the procedure was repeated with the supernatants pooled.

3.9.2 DNA Extraction: Precipitation of Nucleic Acids

The bead-beating was followed by the addition of 260 μL 10 M ammonium acetate to each tube of the pooled supernatant and samples were incubated on ice for five minutes before centrifuging at 4°C for 10 minutes to precipitate and remove the SDS and other impurities. The supernatant was carefully removed and was transferred to two 1.5 mL Eppendorf tubes and 600 μL of isopropanol added. These were incubated on ice for 30 minutes before centrifuging at 16,000x g at 4°C for 15 minutes.

The isopropanol precipitation step served to recover the nucleic acids. The supernatant was removed via aspiration leaving a nucleic acid pellet, which was then washed with 70% ethanol and dried under a vacuum for two minutes. The pellet was then dissolved in 100 μL of Tris-EDTA (TE) buffer and the two aliquots were pooled.

3.9.3 Genomic DNA Purification

To remove RNA from the resolubilized pellets, 2 μL of DNase-free RNase (10 mg/mL) was added to each set of the pooled aliquots and incubated on ice for 15 minutes. Next, the QIAamp® DNA Stool Mini Kit (Qiagen), which provided proteinase K and a
series of buffers and specialized columns, was used for DNA purification. Fifteen (15) μL of proteinase K and 200 μL of Buffer AL were added to the pellets, mixed well and incubated at 70°C for 15 min. To that, 200 μL of ethanol was added and mixed, and then transferred to the QIAamp columns and centrifuged at 16,000 g for one min. The flow-through was discarded and 500 μL of Buffer 1 (AW- alcohol wash-1) was added to the column and centrifuged (16,000x g) for one minute at room temperature. The flow-through was again discarded, and 500 μL of Buffer 2 (AW2) added to the column and again centrifuged (16,000x g) for one (1) minute at room temperature and the column was then dried by centrifugation for one (1) minute at room temperature and 200 μL of Buffer AE (elution buffer) was added to the column (with collection tube attached). After incubating at room temperature for two (2) minutes, the columns were centrifuged at 16,000x g for one (1) minute to elute the DNA. The DNA was then aliquotted into four (4) microcentrifuge tubes. DNA quality was determined by 0.8% agarose gel electrophoresis for each set of DNA extractions. Samples of DNA were stored at -20°C until thawed for PCR and DGGE.

3.9.4 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was used to amplify the Variable 3 (V3) region of 16S rDNA using primers to conserved regions of the 16S rRNA genes (172). The particular forward primer employed was 357f (5'-CCTACGGGAGGCAGCAG-3') and the reverse primer was 519r (5'-ATTACCGCGGCKGCTGG 3') (171). Attached to its 5'end, the 357 forward primer has a 40-base GC clamp attached to it. A PTC-100
Peltier Thermocycler (MJ Research, Waltham, MA) was used to perform the PCR amplifications. The initial amplifications were performed in 50-μL volumes from a fresh master mix comprised of 5 μL PCR buffer (with 20mM Tris HCL, pH 8.4, and 50 mM KCL), 0.4 μL 100 mM dNTP, 0.25 μL of each primer (concentrations 100 μM), 1.75 μL MgCl₂ (50 mM), 1 μL bovine serum albumin (BSA-3.36%), and 0.25 μL Platinum® Taq DNA polymerase, and 36.1 μL autoclaved distilled water, and 5.0 μL extracted DNA (quantified at 20 ng/μL DNA in 30 μL total-DNA plus Tris-EDTA buffer). The Platinum Taq allowed for hot-start PCR. See Table 3.8 for the specific programming of the thermocycler. Negative controls, samples devoid of DNA template, were simultaneously run in the thermocycler. To confirm successful amplification of the V3 region, 8 μL of PCR product from each of PCR reaction and 4 μL of 100 BP ladder were loaded onto a 2% agarose gel (100 mL TAE buffer and 2.0 g agarose) and run at 120 v, 400 amps, for 40 min. Treatment and Baseline samples were run separately on the 2% (w/v) gels. All amplifications were successful in the Baseline sample groups except for subjects #7, 17, and 18; all were successful in the Treatment sample groups except for subjects #6, 13, 16, 23, and 25. It was determined that Tris-EDTA (TE) buffer chelated MgCl₂ from the PCR Master mix, preventing the polymerization of the samples. The MgCl₂ volume in the Master mix was recalculated to change MgCl₂ from 1.75 μL per 50 μL PCR reaction mixture to 2.0 μL of MgCl₂ per 50 μL PCR reaction mixture. This adjustment resulted in successful amplification for the previously chelated samples except for Baseline samples #7 and 17. These two samples were purified again with Centri-Sep columns (Princeton Separations, Inc., Adelphia NJ). The subsequent PCR amplifications were successful.
<table>
<thead>
<tr>
<th>Step</th>
<th>Temp-°C /Time</th>
<th>PCR-Related Function</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>90º/ 0 min</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>94º/ 5 sec</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>94º/ 30 sec</td>
<td>Denaturation</td>
</tr>
<tr>
<td>4</td>
<td>56º/ 30 sec</td>
<td>Annealing</td>
</tr>
<tr>
<td>5</td>
<td>72º/ 45 sec</td>
<td>Elongation</td>
</tr>
<tr>
<td>6</td>
<td>Go to Step 3 X 35 cycles</td>
<td>Final Elongation</td>
</tr>
<tr>
<td>7</td>
<td>72º /8 min</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4° C</td>
<td>Completed</td>
</tr>
<tr>
<td>9</td>
<td>End</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.8 Thermocycler Program Degradation Gradient Gel Electrophoresis (DGGE)-Variable 3 region (V3) Hot-start PCR

3.9.5 Denaturing Gradient Gel Electrophoresis (DGGE)

Parallel DGGE was performed as described by Yu and Morrison (171) and as modified and further adapted in Dr. Morrison’s lab. (See Appendix H) Briefly, 12 μL of PCR product and 7 μL of 100 bp ladder were resolved in a 7.5% polyacrylamide gel comprised of a 40-60% gradient of chemical denaturants (40-60% v/v formamide and 7 M urea (See recipes in Appendix H). The parallel DGGE gels were run at 60 °C for 19h at 73 volts to yield 1387 volt h using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). A water bath was employed and maintained at 60°C instead of the D-Code tank to prevent gel “smiling” on the back gel often due to uneven heating. After running the allotted time, the gels were stained with GelStar® (Cambrex, Rockland, ME). The gels were then transferred to a FluorChem® Imager (Alpha Innotech, San Leandro, CA) and images were obtained and stored until post-gel processing with the Bionumeric Database (Applied Math, Kortrijk, Belgium) When specific primers and real-time PCR are not employed (as in this study), the use of
dendograms and gel composites may be used to display and subjectively discuss the data. A dendogram is a set of all gels placed in a certain order of band similarity via computer generation. A gel composite is one where the scientist manipulates each gel in a logical manner (for this study, by patient and visit) on one sheet for purposes of discussion.

3.10 Nutrient Intake Records (Diet Records)

During the Screening/Initiation visit and then again before the Baseline visit, subjects were counseled as to the protein and caloric value of the Health Source Soy Protein Shake (150 calories, 26 grams protein) and how to reduce their non-soy daily protein intake by approximately the same gram amount during the Treatment Period, since consuming additional protein which could ultimately lead to weight gain. Once the study commenced, reminder calls were made to each subject one day prior to the recording of the food records for both the Baseline and Treatment Periods.

Subjects kept diet records for four days of each period, i.e., during week two and week five of the study. During those weeks, the subjects kept records on the four days preceding their admission to the GCRC (Saturday, Sunday, Monday, and Tuesday). It had been established that recording dietary intake for four days that include two weekdays and both weekend days provides an accurate representation of the subject’s intake for that week (173). The purpose of the food records was to assess the effect of soy protein intake on the daily non-soy protein intake during the Treatment Period. This was determined by comparing Baseline Period non-soy protein intake with Treatment Period non-soy protein intake. A copy of the Food Record can be found in Appendix I.
3.11 Gastrointestinal Tolerance Symptom Record

Gastrointestinal Tolerance Records were completed the same four (4) days as the diet records. The subjects were instructed to self-report symptoms on an hourly basis using a record sheet (32). The degree to which they experienced the various symptoms (bloating, flatulence, abdominal pain, diarrhea/loose stools, headache) was indicated by placing an “X” on a 5-cm line numbered 0 to 5. The symptom severity had a corresponding number for quantification and statistical purposes: 0 = none, 1 = slight, 2 = mild, 3 = moderate, 4 = moderately severe and 5 = severe (174, 175). A copy of the Gastrointestinal Tolerance Symptom Record can be found in Appendix J.

3.12 Statistical Methods

3.12.1 Primary Response Variable

The primary response variable in this study was the change in soy isoflavone absorption that occurred due to provision of FOS in the Treatment Group. The mean changes for the Placebo Group were to be compared using a 2 independent sample t-test, assuming normal distribution in the changes.

As the distributions of the data for the three isoflavones (daidzein, genistein, and glycitein) were nonparametric, the means of the two independent groups (the FOS Diet Group and the Placebo Diet Group) were tested using the Wilcoxin Rank Sum/Kruskal-Wallis Test for Independent Groups, also known as the Mann-Whitney U test, and the 1-way Test, the Chi Square Approximation. The level of significance was set at 0.05.
3.12.2 Secondary Response Variables

Breath hydrogen values were also analyzed for normal distribution and determined to be nonparametric, thus the means of the FOS Group and the Placebo Group were tested using the Wilcoxin Rank Sum/Kruskal Wallis Test for Independent Groups. The level of significance was set at 0.05. A Diversity Index was calculated for each study subject for each visit to reflect changes in stool microflora. The Diversity index reflects the band number and density for each gel, using the Bionumerics Database (Applied Math-Kortrijk, Belgium). The Diversity Index is a quantitative measure of microfloral ribosomal DNA (rDNA), not a qualitative identifier of microorganisms. The Diversity Index for FOS Group and for the Placebo Diet Group for the Baseline Visit and for the Treatment Visit was analyzed in terms of Least Squares Means. For the β-galactosidase measurements, data were log-transformed to achieve homogeneity of variance. LS MEANS plots were used to determine differences in intake of Protein and Calories from the Baseline and Treatment Periods. The level of significance for all statistical tests was set at 0.05. All statistical tests were performed using the “JMP IN” ® software by SAS.
CHAPTER 4

RESULTS

4.1 Demographics

Twenty-four postmenopausal women were recruited for this clinical trial with twelve randomly assigned to the treatment (FOS) group and 12 to the placebo group. There were no differences between groups in terms of mean age, weight at baseline, height, and body mass index (BMI). Age ranged from 49-66 in the FOS group and from 51-68 in the Placebo group. Baseline weight ranged from 47.9-98.5 kg in the FOS group and 59.5-95.9 kg in the Placebo group. Body Mass Index (BMI) ranged from 18.9-37.6 in the FOS group and 22.7-32.1 in the Placebo group. See Table 4.1
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>FOS Group n=12</th>
<th>Placebo Group n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE</td>
<td>59.1 ±1.6</td>
<td>59.9 ± 1.7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.8 ± 4.6</td>
<td>73.1 ± 2.8</td>
</tr>
<tr>
<td>Height (in)</td>
<td>63.4 ± 0.7</td>
<td>64.8 ± 0.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.5 ± 1.8</td>
<td>28.8 ± 0.9</td>
</tr>
</tbody>
</table>

Table 4.1 Means ± SEM for each demographic characteristic are listed. There were no statistical differences between groups.

### 4.2 Isoflavone Content of Soy Supplement Consumed by Clinical Trial Subjects

Postmenopausal women in both the Treatment Group (Soy + FOS) and in the Placebo Group (Soy + Dextrose) consumed two (2) scoops of Health Source® Soy Protein Shake powder daily during the Treatment Period. Each 22 gm scoop of the Health Source Soy Protein Shake (Ross Products Division, Abbott Laboratories, Columbus, OH) contained 13 gram soy protein and 40 mg of soy isoflavones, for a total daily intake of 26 g protein and 80 mg of soy isoflavones. Subjects mixed the shake powder with eight (8) oz. skim or 2% milk and ingested it at breakfast. Typically, the β-glucoside conjugate form of isoflavones predominates in soy powders (17), and fermented soy consists of more aglycones than glucosides. This was not the case in
Health Source as can be seen in Figure 4.1. Although the β-glycoside, genistin, was actually present in the highest amount, the aglycones as a whole accounted for 57% of the total isoflavones. Forty-three percent (43%) of the product was composed of β-glucosides, with genistin, and daidzin accounting for 31.3% and 11.7% respectively. There was no detectable glycitin in the product. Product from three different Health Source batches (0, A, and B) were given to the subjects, with specific batch dependent on time at which they were enrolled in the study. Figure 4.2 demonstrates the consistency of the inter-product composition via a 3-way overlay report. Calculated isoflavone amounts can be found in Table 4.2.
Figure 4.1 Isoflavonoid composition in Health Source Soy Protein Shake. Aglycones comprised 57% of the product, and Glucosides, 43%.
Figure 4.2 Isoflavonoid composition of test product was consistent across batches.
### 4.3 Urinary Isoflavone Excretion

All subjects had isoflavones present in their urine during the *treatment* phase, indicating compliance with the protocol (176). No subjects had appreciable isoflavone peaks at their *Baseline* visit. Three subjects were not included in statistical analyses due to one of the following: 1) omission of urine preservative (Subject #2); 2) extended antibiotic therapy after study intiation (#10); and subject urine tubes missing visit labels (#14). Subjects #2 and #10 were in the FOS Group, and #14 was in the Placebo Group.

Treatment of samples with $\beta$-glucuronidase-sulfatase enzyme, converts all forms of isoflavonoids to their respective aglycones. The FOS group exhibited a higher 24-hour urinary excretion of daidzein, $(2849 \pm 313 \mu g)$ than the Placebo group $(2705 \pm 405 \mu g)$, but the difference between the two groups was not statistically significant ($P = 0.7782$, see Figure 4.3). Similarly, the 24-hour levels of genistein were higher in the FOS group $(928 \pm 197 \mu g)$ than the Placebo group $(795 \pm 136 \mu g)$ but statistically insignificant ($P =$

---

**Isoflavone amounts per-scoop of Health Source Product**

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>Quantities (mg)</th>
<th>Percent-%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistin</td>
<td>25.04</td>
<td>31.3</td>
</tr>
<tr>
<td>Daidzin</td>
<td>9.36</td>
<td>11.7</td>
</tr>
<tr>
<td>Glycitin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Genistein</td>
<td>19.04</td>
<td>23.8</td>
</tr>
<tr>
<td>Daidzein</td>
<td>16.88</td>
<td>21.7</td>
</tr>
<tr>
<td>Glycitein</td>
<td>9.68</td>
<td>21.1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>80</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>

Table 4.2 calculated isoflavone amounts (mg) of glucosides and aglycones.
Glycitein levels in the FOS group (417 ± 89 μg) also exceeded those in the Placebo group (373 ± 56 μg) but were insignificant (P = 0.8602). Mean excretion of isoflavonoids increased by 5% for daidzein, 17% for genistein, and by 12% for glycitein in the FOS group compared to the respective mean isoflavone excretions in the Placebo group. Total recovery of isoflavones in urine as aglycones was 4.84% for the Placebo group and 5.25% for the FOS group with #11 included in the analysis, and 4.33% in the Placebo group and 5.25% in the FOS group when #11 was excluded from the Placebo group analysis.

It’s important to note the broad ranges for the excretion of each of isoflavones in the FOS and Placebo group, reflecting the inter-individual variability. For the Placebo group, excretion values (μg/24 h) ranged from 1069-5445 for daidzein, 276-1848 for genistein, and 0-632 for glycitein. Likewise, in the FOS group, values ranged from 976-4613 for daidzein, 161-1916 for genistein, and 0-867 for glycitein.

In both groups, daidzein was consistently excreted (reflecting apparent absorption) at greater levels than genistein, though the genistein/glycitein ratios varied between subjects. One subject in each group had no detectable glycitein.
Figure 4.3 Urinary profile (mean excretion- μg/24 h) of isoflavonoid aglycones in urine. Differences between groups were not statistically significant.
Histograms for the individual subjects in both the Placebo and FOS Groups can be seen in Figures 4.4 and 4.5.

Figure 4.4 Urinary content of isoflavonoid aglycones for subjects fed placebo with soy shake at Treatment visit.
Figure 4.5 Urinary content of isoflavonoid aglycones for subjects fed FOS with soy shake at Treatment visit.

HPLC chromatograms for depicting peaks for daidzein, genistein, and glycitein for each subject in both the Placebo and FOS groups can be found in Appendix K. The isoflavones were identified based on spectral profiles of pure isoflavone standards and on retention times established in the literature (165).

Although not statistically significantly different, the values for the quantified isoflavones for Subject #11 (Placebo group) were cause for concern as to whether the patient took additional soy or an additional source of FOS. The values for this subject were 41% higher for daidzein than any other placebo subject (and 18% higher than the next highest FOS subject), 58% higher for genistein than the next highest placebo subject, and 9% higher that the next highest placebo subject. The percent increase in
Isoflavone excretion in the FOS group compared to the Placebo group was 5% greater for daidzein, 17% greater for, and 12% greater for glycitein when subject #11 in included in the statistical analysis, and differences between groups were not statistically significant. With the values of Subject #11 excluded, the percent increase in isoflavone excretion in the FOS group compared to the Placebo group increased 1-2 fold in all three isoflavone categories: daidzein 17%, genistien 34%, and glycitein, 20%. However, differences between the Placebo and FOS groups for isoflavone excretion remained insignificant.

Figures 4.6 and 4.7 portray the differences in mean isoflavone excretion with and without the inclusion of the values for subject #11.

![Urine IFN excretion ug/24h](image)

Figure 4.6 Histogram with subject #11 included
Figure 4.7 Histograms with Subject #11 excluded.

Creatinine clearance (an indicator of glomerular filtration rate and hence kidney function) was performed on all subjects’ urine samples, yet there were no significant differences between groups. (Data not shown)

4.3.1 Equol Excretion

The decision to measure equol was made after the protocol was completed, and the pertinent technology became available. Briefly, five (5) of the subjects (23.8%) were equol producers, as defined in this context by that which was detected in the urine of study subjects via HPLC-MS/MS. (See section 3.6). Urine content of equol for subjects 3, 5, and 20 in the FOS group were 3.86, 9.16, and 1.51 μmol/L, respectively. Subjects #8 and 17 in the Placebo group excreted 5.92, and 2.09 μmol/L, respectively. There were no correlations between equol status and breath hydrogen, β-galactosidase activity, diversity index, age, methane status, or caloric, protein, carbohydrate or fat intake. There were
trends upon which to remark for body mass index and urine daidzein. See Chapter 5 – Conclusions/Discussion.

4.4 Non-Soy Protein Intake

The degree of change in daily non-soy protein intake from the Baseline visit to the Treatment visit was evaluated. The protein intake of both groups declined markedly (Figure 4.8). There was an observed difference in the FOS group of -2.959 ($P = 0.0073$); in the Placebo group of -3.287 ($P = 0.0034$). The difference over time was highly significant ($P = 0.005$) and the Group X time interaction was not significant.

![DAILY NON-SOY PROTEIN INTAKE](image)

**Figure 4.8** Daily non-soy protein intake declined significantly in the Placebo ($P = 0.0034$) and FOS ($P = 0.0073$) groups from Baseline (BL) to the Treatment (TRT) visit
4.5 Caloric Intake

Caloric intake analysis was also important, since attempts to decrease protein intake may inadvertently increase carbohydrate or fat intake. A t-test of the pre- and post means using LS Means showed that caloric intake decreased in both groups, but was only significant in the FOS group. The mean estimate of the observed difference in the FOS group was -372 kcals/day ($P = 0.002$); and for the Placebo Group was -163.6 kcals/day, approaching significance ($P = 0.0584$). The effect of time was significant ($P = 0.0001$) but the Group X time interaction was not significant ($P = 0.0843$). See Figure 4.9.

![Graph showing non-supplement daily intake](image)

**Figure 4.9** Non-supplement caloric intake declined significantly in the FOS group from Baseline (BL) and Treatment (TRT) visits but not in the Placebo group
4.6 Breath Hydrogen

All subjects participated in the breath hydrogen collections to determine intestinal gas production. The results for two subjects in the Treatment group could not be used because Subject #7 became nauseous during the testing period and was unable to produce adequate end-alveolar samples and the sample collection period for the 8AM, 10AM, Noon, and 2PM were characterized by technical error as indicated by the suboptimal CO₂ percentage in each sample for the four time points for subject #13.

It was not possible to calculate area under the curve, as technical errors occurred for 15 subjects during the 8AM-2PM four hour sampling period, resulting in the collection of NON-end-alveolar air. Thus only mean adjusted peak hydrogen levels could be calculated. Post-challenge mean adjusted peak hydrogen levels (± SEM) for mid AM (10 AM or Noon) in the FOS Group (46.6 ppm ± 11.1) were statistically significantly higher (P = 0.0001) than in the Placebo Group (3.73 ppm ± 1.29). Figure 4.10.

Five of the subjects were methane producers: Subjects #16, and #22 from the FOS group and Subjects #11, 17, and 18 from the Placebo group. There was no correlation between mean adjusted peak hydrogen levels and methane levels, or between any other study outcome measure or demographic characteristic and methane level.
Figure 4.10 Elevated mean adjusted peak breath hydrogen levels for the FOS group. The asterisk above the error bar indicates that the breath hydrogen for the FOS group was statistically different (P = 0.0001) from the Placebo group.

4.7 β-galactosidase Fecal Enzyme Activity

β-galactosidase activity was measured as an indicator of the directional change in the numbers of *Bifidobacterium*/Lactobacillus/other bacteria during FOS supplementation. Data for β-galactosidase were log-transformed to achieve normal distribution. The Effect Test demonstrated that the Group X Visit interaction was significant at $P = 0.0106$. A post-hoc test of the pre- and post means was performed using the Least Squares (LS) means of the natural log (ln) of the β-galactosidase. See Figure 4.11. β-galactosidase activity (ln) is expressed as LS means in μmol of o-Nitrophenylβ-D-galactopyranoside (ONPG). The change from the Baseline Visit to the Treatment Visit in the FOS group was significant (P = 0.0237); whereas the change from
Baseline to Treatment in the Placebo group, albeit in the opposite direction (β-gal activity decreased) was not significant ($P = 0.1457$).

Figure 4.11 Stool β-galactosidase activity increased after feeding FOS + SOY for three weeks. The asterisk above the FOS Treatment (TRT) group indicates a statistically significant increase ($P = 0.0237$) from Baseline (BL). LS Means ± SEM for FOS Baseline visit, 5.66 ± 0.23; FOS Treatment visit 6.02 ± 0.23, and Placebo Baseline visit 6.02 ± 0.22; Placebo Treatment visit, 5.8 ± 0.22.

4.8 Molecular Characterization of Stool Samples

Following the disk storage of gel photos taken by the Alpha Innotech Fluorchem® Imager, these images were copied to the Bionumerics Database (for post-gel processing) to obtain dendograms and to compute diversity indices.
4.8.1 Dendograms

Once copied to the Bionumerics Database, “fingerprints”, or definitive files, were created for each gel. Four steps then followed, including 1) Gel strip definition (defining lanes); 2) Curve adjustment (adjusting settings for background and computing signal/noise ratio); 3) Normalization (setting external and internal standards using the 100 bp ladder); 4) Bands (Band identification on the gel by the program). After these steps were completed for each gel, dendograms could be created with the compilation of all 48 gel strips. Dendograms were generated to detect any patterns in the subjects’ treatments and/or visits (FOS vs Placebo treatments and Baseline vs. Treatment visit). The dendogram of all 48 gels from the five (5) gel plates are found in Figure 4.12.

Using the dendogram, there is no distinct pattern regarding the effect of the FOS treatment. In fact, three (3) of the subjects were paired together (baseline and treatment visit side-by-side) by the dendogram program (#19, 22, and 24) indicating that many of the banding patterns matched closely between the Baseline and Treatment periods for each of these three (3) subjects. The dendogram also displays sets of gels where several gels in a row are visit-specific; e.g., gel strips for the Treatment visits are clustered toward the top and middle of the dendogram; whereas Baseline visit gel strips are clustered toward the bottom.
Figure 4.12 Dendogram of all 24 subjects’ Baseline and Treatment Visits. (Red = Placebo, Blue = FOS)

4.8.2 Gel Composite

In terms of individual gel presentation, the gels were manipulated by computer to organize them by subject, with each subject’s Baseline visit gel positioned above the Treatment visit gel. (Figure 4.13). Upon examining this organized composite, it became
apparent that each subject had a relatively stable banding pattern (Baseline and Treatment visit), although several subjects in the FOS group appeared to have responded to the prebiotic (the fructooligosaccharide) when comparing the Treatment gel to the Baseline gel. These subjects were #5, 7, 12, 16, 20, 21, and 22. Similarly, several subjects in the Placebo group appeared to have fewer and less dense bands in the Treatment period compared to the Baseline period (subjects #9, 18, 23, 24, and 25). These directional changes were what would have been expected if it was true that FOS would increase band number and density. Conversely, one subject (#3) in the FOS group appeared to have responded in the opposite direction of that which may be expected (fewer bands/less dense bands in the Treatment Period); however subjects # 1, 4, 6 and 8 seemed to have more and denser bands in the Treatment Period, despite being assigned to the Placebo treatment.
Figure 4.13 Gel composite. (Red = Placebo, Blue = FOS) The gels were manipulated by computer to organize them by subject, with each subject’s Baseline visit gel positioned above the Treatment visit gel.
4.8.3 Diversity Index (DI)

Another parameter with which to assess change from the Baseline Period to the Treatment Period is referred to as the Diversity Index (DI), obtained from the Bionumerics Database (Applied Math, Kortrik, Belgium). The dendograms conveyed more subjective information about the gels. For more quantitative information, the DI was used. This index was obtained by exporting the gel information from the Numerics Database (using the “Band/Export File”) and copying the corresponding numeric information for each band to Excel. Specific equations in the program address band number and density. These numbers reflected normal position of the band, height, Relative 1D surface, (in terms of percentage), and the equations incorporated Pi, -lnPi, and (Pi x (-lnPi). Table 4.3 shows the mean DI for each visit (Baseline and Treatment) and for each Group (FOS and Placebo), as well as mean band numbers.
<table>
<thead>
<tr>
<th>Patient Group/Visit</th>
<th>Mean # Bands</th>
<th>Mean Diversity Index</th>
<th>Diversity Index Mean Difference (TRT-BL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo Group – Baseline Visit</td>
<td>21.08</td>
<td>2.94</td>
<td></td>
</tr>
<tr>
<td>Placebo Group – Treatment Visit</td>
<td>21.83</td>
<td>2.95</td>
<td>0.015</td>
</tr>
<tr>
<td>FOS Treatment Group – Baseline Visit</td>
<td>18.82</td>
<td>2.80</td>
<td></td>
</tr>
<tr>
<td>FOS Treatment Group – Treatment Visit</td>
<td>23.36</td>
<td>3.02</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Table 4.3 Mean band number and mean Diversity Index (DI) for each treatment group (Placebo and FOS) at each visit (Baseline and Treatment). The DI difference (TRT-BL) in the FOS Group is statistically significant (P = 0.036). See also Figures 4.14 and 4.15.

The increase the DI from the Baseline Period to the Treatment Period in the FOS Group was different than the change that occurred in the Placebo Group. The difference in DI from Baseline and Treatment in the FOS group was significant (P = 0.036), whereas the increase in the Placebo group was not significant (P = 0.886).

An Effect test revealed that a Group X time interaction was not significant (P = 0.1449). However, a post-hoc t-test of the Least Squares (LS) Means revealed a statistically significant increase in DI in the FOS group. (See Table 4.3, and Figures 4.14 and 4.15).

A bivariate fit of the β-galactosidase (β-gal) difference by the Diversity Index difference resulted in a Pearson’s correlation coefficient, r = 0.7357. See Appendix L for
individual gels with *Baseline* (BL) visit gels and *Treatment* (TRT) gels for each subject, along with the Diversity Index difference (TRT-BL) and the β-gal difference for each subject.

![Graph showing Stool Diversity Index (D.I.)](image)

**Figure 4.14** Stool Diversity Index at Baseline (BL) and Treatment (TRT) for the FOS and Placebo (PLA) groups. The difference in DI from *Baseline* and *Treatment* in the FOS group was significant (*P* = 0.036), as indicated by an asterisk.
Finally, the Gastrointestinal Tolerance Scale Scores revealed that the soy, FOS, and dextrose placebo were generally well-tolerated. Of the six designated descriptors associated with numbers, (0 = none, 1 = slight, 2 = mild, 3 = moderate, 4 = moderately severe, and 5 = severe, only the first four were used. The categories rated were abdominal pain, bloating, flatulence, diarrhea, and headache. Even though 1 subject in each group had diarrhea one time, they each attributed it to a gastrointestinal virus. Of the headaches reported by 5 subjects, all were attributed to sinus or something other than the study products. Table 4.4 provides a statistical breakdown of the severity of the three symptoms that may have been attributed to FOS or Placebo (Dextrose.) There were
virtually no difference between the two groups in the categories of abdominal pain, bloating and flatulence, and there was no study attrition. All subjects stated they would participate in such a study again, and 30% continued taking FOS on their own after the study was completed.

<table>
<thead>
<tr>
<th>GASTROINTESTINAL TOLERANCE SCALE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FOS group</strong></td>
</tr>
<tr>
<td><strong>Abdominal Pain</strong></td>
</tr>
<tr>
<td>75% None</td>
</tr>
<tr>
<td>25% Slight</td>
</tr>
<tr>
<td>0% Moderate</td>
</tr>
<tr>
<td><strong>Bloating</strong></td>
</tr>
<tr>
<td>66% None</td>
</tr>
<tr>
<td>17% Slight</td>
</tr>
<tr>
<td>17% Moderate</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Flatulence</strong></td>
</tr>
<tr>
<td>25% None</td>
</tr>
<tr>
<td>50% Slight</td>
</tr>
<tr>
<td>8% Mild</td>
</tr>
<tr>
<td>17% Moderate</td>
</tr>
</tbody>
</table>

Table 4.4 Percentage of subjects reporting gastrointestinal symptoms during the clinical trial. There were no differences between the FOS and Placebo groups.
I conclude that this randomized clinical trial demonstrated that the provision of FOS to postmenopausal women alters gut microbial metabolism. This alteration results in a trend where the apparent absorption of the isoflavones in postmenopausal women is mildly enhanced as measured by urinary isoflavone excretion. The breath hydrogen levels of those consuming the FOS rose significantly, indicating short-term FOS-mediated alterations in the gut microflora. The significant rise in the β-galactosidase activity from the Baseline to the Treatment period also suggests an increase in total resident bacteria. Accordingly, the rise in the diversity index (DI), a measure of bacterial number and density, as well as the positive correlation between the DI and the β-galactosidase activity, may also serve to suggeste that the increase in microbiota could have been comprised, in part, of *Bifidobacterium* and *Lactobacillus*, among others. However, it’s crucial to emphasize that differential techniques (specific primer and real-time PCR) are required for definitive statements regarding the genus and species of comprising the increase in the microbiota.

The protein and caloric data from the food records unequivocally demonstrate that this group successfully adhered to diet instructions for the modification of their intake to account the dietary contribution of soy to additional protein and calories.
Lastly, The Gastrointestinal Tolerance Records revealed that both the soy and FOS at the doses provided were well-tolerated.

To summarize the conclusions, the increase in the total resident bacteria as evidenced by the statistically significant rise in the Diversity Index, the β-galactosidase activity, and adjusted peak breath hydrogen underscore the magnitude of the physiological effects of FOS and soy on the microflora of postmenopausal women. Thus this study provides a hypothetical model on which to base the design for further research to address the effects of soy and/or prebiotics on the microbiota in this population.

Following are items of discussion.

### 5.1 Urine Isoflavones

The results for the individual urine isoflavones in this clinical trial are congruent with observations cited in earlier related literature that inter-individual variations characterize the study of bioavailability of soy isoflavones (133, 150-154). Subject #11, a conscientious elementary school teacher, was randomized to the placebo group and was compliant with study protocol. Urine isoflavone values (μg/24h) for this subject were daidzein - 5445, genistein - 1848, and glycitein - 632. Of note is that these values far exceed even the next highest value for any of those in the Placebo group; for daidzein, by 41%; for genistein, by 58%; and glycitein by 9%. Her values even exceed the highest daidzein value in the FOS group by 15% (but not the highest genistein or glycitein value in the FOS group). There is not a ready explanation for this, as can be seen from Appendix L, where the Treatment gel does not appear to be more dense in terms of bands
The DI is only 0.089, and the change in β-galactosidase from the Baseline to Treatment period is negative (-105 μmol ONPG split/g stool). One note of interest is that this subject was one of the five methane producers, and interestingly, exhibited the highest methane production of those five subjects.

Another item of note is that even though the composition of the Health Source product was 55% of the genistein family (genistin and genistein) and 33% of the daidzein family (daidzin and daidzein- Table 5.1), daidzein appears to be consistently absorbed at greater levels than genistein as measured by urine isoflavones in all subjects. This concurs with results found in rat (177, 178) and human (179-182) studies where daidzein absorption is 2-3 times greater than that of genistein. It has been suggested that the lesser amount of excreted genistein in the urine compared with daidzein may be attributed to differences in molecular structure and differential metabolism (154,182,183). It was reported over 30 years ago that genistein has a greater susceptibility to microbial breakdown than daidzein or glycitein. Genistein is characterized by the hydroxyl group at the 5 position of the A-ring, and this is degraded more readily by gut microbes than are compounds devoid of this structure, such as daidzein and glycitein. Thus greater microbial degradation is associated with lesser amounts recovered in the urine (182).

Contrasting opinions exist in the literature as to whether the bioavailability of isoflavones (aglycone vs glucoside) differ. It has been shown in several pharmacokinetic studies that aglycones are absorbed more rapidly than are glucosides, due to the need for deglycosylation of the glucoside prior to absorption. Setchell et al. (154) studied the
bioavailability of pure isoflavones (in aglycone and glucoside forms) and reported that the mean times to achieve peak plasma concentrations ($t_{\text{max}}$) for the aglycones, genistein, and daidzein were 5.2 and 6.6 hours, respectively. However, peak plasma concentrations for the glucosides, genistin and daidzin, showed that they were not absorbed until 9.3 and 9.0 hours, respectively, consistent with deglycosylation of the glucoside. The plasma area under the curve (AUC) was higher following consumption of the glucoside as opposed to the aglycone. They speculated that the greater bioavailability of the glucoside isoflavones can be attributed to the protection the glucose group affords the molecule from biodegradation. Richelle et al. (184) found that supplementing postmenopausal women with hydrolyzed isoflavone glucosides did not affect plasma and urine isoflavone pharmacokinetics when compared with non-hydrolyzed isoflavone glucosides. This observation was confirmed by Zubik and Meydani (185) who reported similar findings when aglycones and glucosides were given in isoflavone supplement form. The observations of Izumi et al. (186) differ from Setchell et al. (154) who found that glucosides were more bioavailable than aglycones (in premenopausal women) and Richelle et al and Zubik and Meydani who found no difference in bioavailability between glucosides and aglycones, primarily in Caucasian women. Izumi et al. (186) reported that the bioavailability (AUC) of the aglycones genistein and daidzein was significantly higher and $t_{\text{max}}$ was faster than for the glucoside forms in Japanese women. With the conflicting results regarding the bioavailability of aglycones vs glucosides, one must keep in mind that a multitude of variables is involved: ethnic background, habitual diet, food matrix, number of subjects, and of course, gut microflora. Despite the conflicting
findings, many agree that the aglycone form of isoflavones are absorbed more rapidly, although the isoflavone glucosides are absorbed to the same degree. Thus, bioavailability of aglycones and glucosides as reflected by total urinary isoflavone excretion appears to be independent of the ingested form based on recent papers (182-185). The fact that the Health Source™ Soy protein shake had a higher percentage of glucosides is most likely a non-issue in regards to total absorbed, however, one must consider the amount of FOS that reaches the intestine for it’s probable contributory role in the production of bacterial metabolites of isoflavones.

5.2 Breath Hydrogen

Bifidobacteria and lactobacilli are not known to produce hydrogen during metabolism of prebiotics, yet the use of breath hydrogen analysis with the administration of FOS is not typically employed. It was used, however, in a recent Master’s Thesis where a 40% decrease in area under the curve for breath hydrogen occurred (187) It can be speculated that perhaps the beneficial non-hydrogen producing bacteria (Bifidobacteria and lactobacilli) contribute to initial, partial metabolism of FOS (and perhaps soy constituents), and then other hydrogen-producing strains carry on the metabolism, resulting in hydrogen production. Theoretically, this partially metabolized FOS may then be metabolized again by the beneficial bacteria, ultimately leading to increased bacteria number. This exciting area involving a synergy between two groups of bacteria requires further elucidation.
5.3 β-galactosidase Activity

Fructooligosaccharides (FOS) are known to specifically increase the population density of *Bifidobacteria* (188-190) and β-glucosidase activity in the large intestine (191). FOS has also been shown to increase the number of lactobacilli when conditions (pH, duration of FOS supplementation) are optimal (192). Several studies report that *Bifidobacteria*, *Lactobacillus*, and *Bacteroides* possess β-glucosidase activity (147,179,192). Our lab has found that the administration of FOS also increased the β-galactosidase activity by 50% (187). Therefore, fecal β-galactosidase could be considered an indirect indicator of change in the *Bifidobacteria* and/or *Lactobacillus* activity. One caveat with the results is that the stool was frozen for three to six months, thus the total enzyme activity may have been compromised. T

Truly conclusive statements can only contain references to increases of β-gal activity as reflecting an increase in total bacterial number, since specific primers and real-time PCR were not employed in this study. This is one of the few studies conducted to date to use both conventional stool enzymes and molecular work to characterize the gut microflora, and as stated earlier, a strong correlation between the two was found.

5.4 Fecal Isoflavone Degradation Phenotypes and Gut Transit Time

Although results from this study regarding the effect of treatment with FOS on urine soy isoflavone concentrations and the molecular characterization of the fecal samples provide compelling results indicating definite trends of change in the increase of general microbial numbers and band densities, there are other items for consideration.
Zheng et al (182), described the prior stratification of subjects in isoflavone bioavailability studies based on fecal degradation phenotype and gut transit time (GTT). They first determined fecal degradation phenotype using an *in vitro* fecal anaerobic incubation. GTT was determined by the provision of 16 ceramic marker beads with the isoflavone powder treatment (182). In this elegant study, they found that subjects with high daidzein degradation rates also had longer (slower) GTT and lower urinary isoflavone excretion rates. These subjects had lower urinary isoflavone excretion rates, whereas subjects who had low daidzein degradation rates had shorter (more rapid) GTTs and these subjects had higher urinary isoflavone excretion rates. Interestingly, the women with the highest urine equol levels in my study also had had low urine daidzein concentrations. In my study, 24% of the subjects were equol-producers (E-P), consistent with the literature (194). The mean urine daidzein for all placebo subjects was $2706 \pm 405 \, \mu g/24 \, h$. The mean urine daidzein for the two placebo E-Ps was $1129 \pm 61 \, \mu g/24 \, h$. The two subjects with the lowest urine daidzein in the entire placebo group were the equol producers. Likewise, the urine daidzein for all FOS subjects was $2847 \pm 313 \, \mu g/24h$ and the mean urine daidzein for the 3 FOS E-P was $2705 \pm 127 \, \mu g/24h$.

Thus our results are in line with the argument presented by Zheng et al. (182). In our study, we also found that the women with the lowest body mass index (BMI) were equol producers. It would be interesting to see if GTT and fecal degradation rate correlated in future studies with equol-producing status.
5.5 The Fate of Ingested Isoflavones and the Role of Gut Microflora

Setchell et al. (17) and many others have done much to elucidate the fate of the ingested isoflavone. Briefly, the isoflavone can be ingested as a glycoside or an aglycone. The glycoside must first undergo enzymatic cleavage of the glucose moiety. The aglycone is generally glucuronidated in the enterocyte or liver, absorbed as a non-conjugated aglycone (small amount) or it is converted to other metabolites by intestinal bacteria. See Appendix M for a detailed schematic and explanation of the fate of the isoflavone.

It is clear from earlier work (17, 133) that there are three sources from which the \(\beta\)-glucosidase for cleavage of the glucoside isoflavone to the aglycone may originate: 1) the small intestinal cell (enterocyte) cytoplasm; 2) on the enterocyte brush border membrane (BBM), and 3) from bacteria, specifically, \textit{Bifidobacteria}, \textit{Lactobacillus}, and \textit{Bacteroides}. Obviously, FOS would have affected only the bacterial enzyme levels, and herein lies the discrepancy in the recent accepted explanations regarding the fate of the ingested isoflavone and the residential location of the bacteria. Cassidy and Setchell (130) state that the isoflavone glucosides are in fact hydrolyzed by \textit{both} mucosal and bacterial glucosidases, but their references for small intestinal involvement clearly refer to \textit{mucosal} (as opposed to bacterial) \(\beta\)-glucosidases. Accordingly, a well-designed study conducted with ileostomates (164) concluded that since the subjects had no detectable bacterial metabolites of soy (dihydrodaidzein, dihydrogenistein, equol, and/or \(\alpha\)-desmethlyangolensin) in their urine, “small intestinal \textit{mucosal} enzymes were primarily responsible for the hydrolysis of isoflavone glucosides. Some of the bacterial metabolites
were detected in control subjects with an intact colon (164). Turner et al speculate that the bacterial involvement in the release of the aglycone from the glycoside in the small intestine is major, with the contribution of nonbacterial enzymes being relatively minor (133). They then delineate the predominance of various microbes in the small intestine and underscore the “obvious implications for deglycosylation and uptake of aglycones in this region (133).” They provide provocative arguments as to why the region of absorption of phytoestrogen and their metabolites is most likely the small intestine, and that the view held by many (154, 178, 195), that isoflavones are metabolized in and absorbed in the colon because of the perceived numbers of microflora (in the gut) is erroneous (133).

The above discussion has implications for our study, and obviously these concepts were not taken into consideration when our trial was designed in 2000. The pre-clinical trial stratification of subjects based on fecal degradation phenotype and GTT status (182) would in effect provide a screening tool to identify subjects who would excrete more urine isoflavones than others. Not screening for this (as in our study) renders the subjects more heterogeneous, and can offset positive results associated with increases in microbial density. Conversely, the assertions by Turner et al. (133) that small intestinal microbial activity is more dominant than most comprehend, may also affect study designs in the future. Perhaps, as for subject #11, in our study the concepts above would serve to elucidate the answer to the question as to why apparent urine isoflavone absorption was so high in some subjects; although the corresponding values for diversity index and fecal \( \beta \)-galactosidase were not necessarily congruent.
CHAPTER 6
EPILOGUE

Our study provided appreciable data regarding physiological and molecular indicators of change in the postmenopausal woman consuming soy and FOS. As with any study, retrospective analysis with the benefit of results from more recent studies and scientific review papers provided suggestions for improved clinical trial design and outcome measures. Some study limitations were predicated on resource allocation, specifically time and financial resources, or simply deemed to be beyond the scope of the doctoral plan of study. The following suggestions preclude any concern about these considerations.

6.1 Isoflavone and FOS Dosages

The treatment dose of 80 mg isoflavones per serving could have been modified to 30-50 mg, considered a more usual intake than a dose of >50 mg/day (154,196), thus a possible cause for concern based on animal studies if taken over extended periods of time (197). However, the mean body weight of our subjects was 70.9 kg, thus 80 mg/d provided only 1.128 mg soy isoflavone per kg of body weight which is consistent with doses found in the literature and not likely to cause any safety problems (198).
Conversely, the dose of FOS could have possibly been higher, yet intolerance of doses >9 g FOS per day could be an issue; and doses of just 15 g FOS per day could cause significant symptoms of abdominal pain, eructation, flatulence, and bloating compared to those taking sucrose (199). A study in rats has alarmed some due to the effect of FOS on modifying (increasing) intestinal permeability leading to salmonella translocation (200). However, those animals were given 60 g FOS/kg of body weight, and our study subjects received 0.13 g FOS/ kg body weight, a negligible amount in the context of the rat study.

The use of galacto-oligosaccharides (GOS) is gaining in popularity in various infant trials designed to study the effects of prebiotics on bifidobacteria, and it appears to be even more bifidogenic than FOS (201). When I designed my trial, GOS was not widely studied or available.

The challenge in clinical trials is to provide the most efficacious interventions dosing with the least amount of safety concerns for the patients or problems with discomfort leading to high attrition rates. There was no attrition in this clinical trial; all subjects who passed the screening visit remained on the study for the five (5) week duration.

6.2 Urine Collections

A 48-hour urine collection may have provided more accurate numbers, but the literature varies on this issue. In well-controlled studies stratifying for phenotypes (182), even the first 12 hours of urine collection has a high correlation coefficient (r = 0.97;
with the second 12 hours, but some isoflavones (for example, equol) can be detected in urine up to 36-48 hours from ingestion (171). Thus, equol is most likely underestimated or undetected in some samples. Since the equol was a post-hoc, post-study design addition to the outcome measure (it was not in the protocol, but the technology to analyze the metabolite became available after the urine was collected and frozen) samples were run for preliminary information only.

6.3 Molecular Indices of Changes in Microbiota

Ideally, time and resources would have been available to differentially identify bacterial strain by the use of genus-specific PCR primers (172, 202). Instead, universal primers were used and diversity indices, reflecting band number and density, were computed.

6.4 Number of Study Subjects and Clinical Trial Design and Duration

Although statistical power would have been greater than 85% with a larger number of subjects, it should be noted that this was considered a pilot study. Also, the conduct of a clinical trial is very expensive and, gratefully, this study was run on The Ohio State University’s General Clinical Research Center (GCRC) with full nursing support and NIH funding, alleviating a huge cost concern for this project. Thus, the achievable number of subjects and GCRC support made it possible for the primary author and co-investigator/RD of this study (a doctoral student) to perform the clinical portion of the study without additional funding. As for study duration, the original protocol and IRB
approval was based on an 11-week, cross-over design, written by the same co-
investigator. After contacting 100 subjects, only one woman consented to the study; all
others declining because the 11-week time commitment was too great. Thus, the protocol
was rewritten as a 5-week, parallel, randomized clinical trial, and subject recruitment and
clinical trial completion then took only 13 months. Many soy bioavailability studies in
the literature are based on one bolus dose only, and longer term supplement feeding is not
investigated, nor are food records generally kept, as in this study.

6.5 Suggested Clinical Trial Design

Future clinical trials should further investigate the metabolism of the isoflavone
and the role of gut microbiota by employing tracers to assist in differentially identifying
the fate of the aglycone. Ileostomy studies provide keen insights as to metabolism and
apparent absorbption in the intestinal compartments. Stratification as to GTT and fecal
degradation status will serve to provide tighter control of the many confounding variables
characterizing this type of study. Because isoflavones alone can stimulate microbes in
the Clostridium coccoides-Eubacterium rectale cluster, Lactobacillus-Enterococccus
group, Faecalibacterium prausnitzii subgroup and Bifidobacterium (203), it would be
desirable to have a study design where the pre-SOY and post-SOY assays are conducted
with real time PCR (specific primers) and DGGE to differentially identify the
contributions of Soy and of FOS to the microflora. No placebo carbohydrate powder
would be given to this group so as not to further confound the microfloral growth.
Limitation of calories in form of carbohydrate would be addressed so as to not affect equol production. The other study arms would be the same as in the current study.

Soy protein isolate (with isoflavones), which appears to be safe from the plethora of studies conducted, should be studied further (as opposed to pure isoflavone extracts) to further elucidate the health benefits imparted by this celebrated bean.
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APPENDIX A

BIOMEDICAL FORM
IRB meetings are scheduled on the 2nd and 4th Mondays of each month (see schedule for exceptions). Applications are processed in the order in which they are received; early submission is recommended. Research requiring review by the fully convened IRB is reviewed approximately 3-4 weeks following receipt of complete applications. Minimal risk research qualifying for expedited review procedures is reviewed outside of the fully convened IRB and will be completed throughout the month. Only applications that are complete will be scheduled for IRB review. Incomplete, inaccurate, or inadequately prepared submission materials will be returned to the principal investigator. *Schedule is subject to change or cancellation.

Principal Investigator: Anne M. Smith, PhD, RD, LD

OSU Faculty or approved PI status

Academic Title: Associate Professor

Phone No. 292-0715

College: Human Ecology, Dept of Nutrition

Department/No.

Campus Address: 325 Campbell Hall

E-mail smith.238@osu.edu

Co-Investigators: Maureen E Geraghty, MS, RD, LD

Protocol Title: The Effect of a Fruit Fiber on the Intestinal Absorption of Isoflavones from Soy Protein in Postmenopausal Women.

Department Chair(s) Endorsement: Mark L. Failla, PhD

Proposed Research Involves:

- [ ] Investigational Drug(s) or investigational use of marketed drug(s). If yes, provide:
  BND Number
  Issued to
  Generic Name:

- [ ] Investigational Devices(s). If yes, indicate NSR or SR; if SR, provide: IDE Number

- [ ] Radioactive Drugs or Unusual Exposure to External Radiation. Approval by the Medical Radionuclide Committee (Phone: 292-0122) is required prior to activation. Investigator is responsible for obtaining approval from both committees.

- Cancer-related activities. All cancer-related protocols must be submitted to the Chair of the James Cancer Center Clinical Scientific Review Committee (Phone: 293-4976) prior to IRB review. For protocols determined by the CSRC chair to be clinical trials, the investigator is responsible for obtaining approval from both committees.

- [ ] Pregnant Women. Approval by Maternal-Fetal committee (Phone: 293-8736) is required prior to activation. Investigator is responsible for obtaining approval from both committees.

- [ ] Minors (Under 18 years of age)

- [ ] Cognitively Impaired

- [ ] Fetuses/In Vitro Fertilization

- [ ] Prisoners
1. Abstract (overview of research)
This pilot study will evaluate the effects of a commercial soluble fruit fiber (called fructooligosaccharides or FOS) on the absorption of some of the active ingredients in soy protein (isoflavones) in postmenopausal women. Fructooligosaccharides (FOS) are naturally occurring polymers of fructose (fruit sugar) attached to a glucose molecule. FOS can also be made commercially by an enzymatic action on sucrose. When FOS are consumed, they act as a food source for the "friendly" bacteria that naturally reside in the colon (large intestine). These friendly bacteria, such as Bifidobacteria and Lactobacillus, make an enzyme that helps breakdown the isoflavones in soy protein. When the isoflavones are broken down, they can be absorbed by the body from the intestines into the bloodstream. It is speculated that when there are not enough friendly bacteria in the colon, soy isoflavones cannot be properly absorbed and used by the body. The consumption of FOS and of soy protein is associated with health benefits. The FOS provided in the study is a commercial product called Nutraflora® (GTC Nutrition LLC, Golden, CO.) and the soy product is Health Source® High Protein Shake Powder, (Ross Products Division/Abbott Laboratories, Columbus, OH). The placebo powder, a dextrose powder, is from Sigma Chemical St. Louis, MO).
This is a prospective, randomized, parallel clinical pilot study. Twenty-four women will go through this five-week protocol.

The study is comprised of two periods and lasts a total of five weeks: 1) The Baseline Period (The first 2 weeks) and 2) The Treatment Period (weeks 3, 4, and 5). A Screening Visit will precede the Baseline Period. At the end of a successful screening, subjects will be counseled by a Registered Dietitian (Co-investigator) to eliminate foods inherently high in soy or FOS (other than the soy and FOS study treatments).

Baseline Period
The Baseline Period is two weeks in duration and begins Week 1 and ends after Week 2. During the Baseline Period, subjects will eat their normal diet. They will be asked to record their dietary intake the four days preceding their admission to the GCRC. During this GCRC visit, subjects will be requested to fast from 6 PM on Wednesday evening until 6:00 AM on Thursday the following day.

Subjects will be admitted to the GCRC on the Wednesday of the second week of the Baseline Period for approximately 20-hours. They are admitted at 6:00 PM and stay until 2 PM the following day (Thursday) to obtain a fecal sample, one (1) breath hydrogen sample, 4 blood samples and a partial 24-h urine sample. The remainder of the 24-hour urine sample will be collected by the subject at home and the Co-investigator will arrange for urine sample pick up at the subject’s home.
Treatment Period

The Treatment Period is three weeks in duration and begins Week 3 and ends after the Week 5 visit to the GCRC. During the Treatment Period, subjects will be randomized to take Treatment A (soy shake and a placebo powder taken in place of the FOS) or Treatment B (soy shake and FOS) every morning for the 3 weeks of the period. The Health Source Soy Protein Shake Powder is made with two scoops of the powder and 8 oz of skim milk. The two scoops of powder provide 26 grams of protein and 80 milligrams of isoflavones. When subjects are on Treatment B (soy and FOS) they will be dosed incrementally with the FOS for a total of a moderate dose of 10 grams per day (2 1/2 teaspoons) by the fourth day of the Treatment Period. This gradual dosing allows for the intestines to adjust to the additional fiber in the diet. Subjects will be counseled to take three divided doses of the FOS to also enhance gastrointestinal intolerance.

Subjects will be asked to record their dietary intake the four days preceding their admission to the GCRC on the Wednesday of Week 5. During Treatment GCRC visit, subjects will be requested to fast from 6 PM on Wednesday evening until 6:00 AM on Thursday the following day.

During the Treatment Period, subjects will be either given Treatment A or B (the treatment to which they have been randomized) at 6:00 AM on Thursday morning. No treatment or other food will be given at 6:00 A.M during the Baseline Period. Samples will be collected until 2 pm that Thursday, specifically, one fecal sample, six (6) breath hydrogen samples, four (4) blood samples and one partial 24-h urine sample. The remainder of the 24-hour urine sample will be collected by the subject at home and the Co-investigator will arrange for urine sample pick up at the subject’s home.

Sample Collection Details

Breath Hydrogen samples will be collected to assess hydrogen production of the bacteria in the colon. The subjects will be asked to breathe normally until they are at the end of a breath (to provide end-alveolar air). At the end of the breath, they will breathe for 3 more seconds into a 60 mL syringe to provide the breath sample. During the Baseline Period, only a 6:00 AM breath sample will be requested. A total of six breath samples will be taken that Thursday of the GCRC admission during the Treatment Period at 5:45, 6:00, 8:00 and 10:00 A.M., 12 noon, and 2:00 PM. During the 4th and 8th hour of sampling, subjects will be allowed to consume 1-2 cans of Ensure Plus® (Ross Products Division, Abbott Laboratories) a product which is known not to contribute to hydrogen production in the colon. Breath hydrogen samples will be analyzed by a Quintron Microlyzer Model SC Chromatograph.

Subjects will also be requested to provide blood samples (5-10 mL) at 8:00 AM and 2:00 PM on the Thursday of the two GCRC visits for plasma selenium and isoflavone levels. (Since isoflavones are phytoestrogens and estrogen and selenium are related, measurements of both blood isoflavones and selenium are desired). A registered nurse will insert an indwelling catheter into the antecubital vein in the subject’s right arm prior to the 8:00 AM blood draws. Subjects will be asked to collect a 24-hour urine from 6 AM Thursday morning to 6:00 AM Friday morning. Urine and plasma isoflavones will be analyzed via established methods using high performance liquid
chromatography (HPLC). At one time point, a fecal sample will be collected at both visits in the GCRC. These samples will be used to characterize the microflora using molecular techniques. Lastly, subjects will be requested to keep 4-day food records and 4-day gastrointestinal tolerance records the 4 days prior to each GCRC admission (Saturday, Sunday, Monday, and Tuesday of the admission week). A registered dietitian will provide these records and counsel the subject regarding their use. The diet records will demonstrate compliance with the treatments as well as other useful nutrition information. The gastrointestinal symptoms records will allow for documentation of possible symptoms the soy and/or FOS may cause along with the intensity and duration of these possible symptoms.

Statistical analysis:
The statistical program “nQuery Advisor” Version 5 was used to carry out the power analysis to justify the sample size. A sample of 12 in each group will have 83% power to detect an effect size of 1.250 using a two sample independent t-test with a 0.50 two-sided significance level. Likewise, a sample size of 11 in each group will have 79% power to detect an effect size of 1.250 using a two sample independent t-test with a 0.50 two-sided significance level. Although this study has not yet been conducted in humans, the “Effect size” of 1.250 is reasonable based on changes seen in animal studies where soy and FOS were administered.

The primary response variable is the change in soy isoflavone absorption that may occur from the provision of FOS in the diet. This will be measured by plasma and urine isoflavone concentrations. The assumed mean change for those receiving Treatment A (Soy + placebo) = 0; the assumed mean for those receiving treatment B (Soy + FOS) is 1.25. An independent sample t-test will be employed for this determination.

The other response variables are as follows and changes will be reported with descriptive statistics (calculations will include the means, standard errors, and confidence intervals and testing for relevant effects and correlations: 1) gut microflora (numbers); 2) β-glucosidase activity; 3) breath hydrogen; 4) plasma isoflavones; and 5) plasma selenium. Subjects’ diet records will be analyzed to assess compliance with study guidelines for daily protein (soy and non-soy) intakes. Gastrointestinal Tolerance records will be comprised of symptoms with a numeric scale that increases with the intensity of the perceived symptom, e.g., a rating of “5” for bloating is worse than a rating of “2.” Differences in symptoms will be evaluated for the two treatments.

The block randomization design (four subjects in the first batch going through the GCRC-two on Treatment A and two on Treatment B, and so on for the next 5 batches of four) will allow us to observe any attrition that may occur and enroll the appropriate number to achieve a total of twenty-four (24) subjects.

The “JMP IN®” software by SAS and the corresponding primer, “A Guide to Statistics and Data Analysis Using JMP® and JMP IN® Software: 4th ed” (by Sall J, Lehman A, Creighton L, Duxbury Thomson Learning, 2001) will be used for statistical analysis.
2. Describe the requirements for a subject population and explain the rationale for using in this population special groups such as prisoners, children, the mentally disabled or groups whose ability to give voluntary informed consent may be in question. Address means of pregnancy screening for females.

Twenty-four (24) healthy postmenopausal will be recruited for this study. Subjects cannot have had a menstrual period within the 12 months before study initiation. Since the women in this study will be postmenopausal, the need for a pregnancy test will be obviated. Subjects cannot be older than 70 years (inclusive) of age. They cannot have taken any medication that would alter lipid, bone, or calcium metabolism, including hormone replacement therapy six months prior to study enrollment. The subject must be free of chronic disease and must not have a history of gastrointestinal or malabsorptive disorders. The subject may not be lactose intolerant. The subject cannot have taken soy products or FOS products, antibiotics or probiotics the three months prior to study enrollment.

3. Describe and assess any potential risks - physical, psychological, social, legal, financial, or other - and assess the likelihood and seriousness of such risks. If methods of research create potential risks, describe other methods, if any, that were considered and why they will not be used.

The risks of the protocol are minor and may be associated with blood sampling, fasting, or with gastrointestinal side effects of the soy shake and/or FOS fruit fiber powder. An attending physician, Dr. Paul Monk, MD will be available to admit subjects to the OSU General Clinical Research Center (GCRC) to assume medical record responsibilities, and to address other medical needs, should they arise. A Registered Nurse will perform the blood draws using sterile technique with the appropriate medical supplies and will exercise universal precautions pertaining to the procedure. Possible side effects of venous blood draws include pain, redness, swelling, bruising, and rarely, an infection or clotting at the indwelling catheter site. Fasting can cause dizziness, headaches, stomach discomfort, or fainting. All blood draws by nursing and fasting by the subjects will be done only while in the GCRC. Possible side effects from the soy shake or the FOS (fruit fiber) include nausea, vomiting, cramping, diarrhea, gas, or bloating. These gastrointestinal side effects may be more likely with the FOS fiber powder than with the soy shake. If the subject experiences discomfort with the 10 grams a day (2 1/2 teaspoons daily) of FOS, the dose can be decreased for tolerance if necessary. Both the Nutraflora® FOS (fructooligosaccharide) powder and the Health Source® Soy Protein Shake Powder are commercially available. The Nutraflora® FOS is the only FOS product in the United States to have GRAS recognition (GRN 000044).

4. Describe consent procedures to be followed, including how and where informed consent will be obtained. (The use of a finder's fee for recruiting subjects is not permitted.) Potential subjects will meet with the co-investigator. The protocol will be explained, questions answered and informed consent obtained in a private room (e.g. exam room in the subject's gynecologists' offices if the patient is identified by one of the gynecologists affiliated with the study. For those subjects responding to advertisements (to be placed on the OSU campus), a private meeting will be arranged either in the prospective subject's office or home, after the subject has been provided information about the study and has successfully met subject eligibility criteria over the phone.
Admission to the GCRC will then be scheduled. One copy of the informed consent, signed by the subject, will be provided to the subject for her files. The other copy of the informed consent will be kept in a locked file cabinet in the primary investigator's office in 347 Campbell Hall.

5. Describe procedures (including confidentiality safeguards) for protecting against or minimizing potential risks and an assessment of their likely effectiveness.

The subject will be in the GCRC for the duration of their fast and for all biological sample collections, with the exception of the last 16 hours of the urine collection. They will be discharged from the GCRC after all blood draws and breath hydrogen collections. Medical personnel will be present or immediately available should the subject feel faint, nauseated, etc., during the fasting time period or during the blood sample collection and breath hydrogen collection time period. All appropriate medical equipment will be used and universal precautions implemented with every subject. Confidentiality will be maintained by using subject initials and numbers instead of full names. All screening and other study information/data will be kept in a locked file drawer in the Co-investigator's office at all times when not in use. Since a portion of this research project will be conducted at the Ohio State University (OSU) General Clinical Research Center (GCRC), the Program/Medical Director, Research Subject Advocate and/or members of the nursing staff, may review medical records. A copy of the written informed consent will be given to the subject for her own records and reference. The Ohio State University Biomedical Human Subjects Review Committee and the United States Food and Drug Administration (FDA) will be granted direct access to study records for verification of clinical trial procedures and/or data, without violating confidentiality, to the extent permitted by the applicable laws and regulations. If the results of this clinical pilot study are published, subject identities will remain confidential.

6. Assess the potential benefits to be gained by the individual subject, as well as benefits which may accrue to society in general as a result of the planned work.

Potential benefits include the attainment of knowledge about soy and FOS in general, and the possibility of adding these two functional foods to their diet on a regular basis. Long term consumption of soy is associated with such benefits as reduced serum cholesterol, reduction of the rate of loss of bone mineral density, and protection from various cancers. Some people have seen an improvement in their serum cholesterol after one month of soy consumption at the study dose. The FDA has approved the statement on all soy products that, "Soy Protein May Reduce Your Risk of Heart Disease" based on a dose of 25 grams of soy protein per day. In this study, subjects will be consuming 26 grams of soy protein a day from the Health Source Product. Along with other nutrients, this soy product also provides 600 milligrams of calcium per serving. Fructooligosaccharides can have a positive impact on overall health (via increasing the balance of friendly bacteria in the bowel) by improving bowel regularity, enhancing calcium and magnesium absorption by the body, and by helping to lower cholesterol. Many of these benefits can be seen after four weeks of consumption of FOS at 4 tsp. per
day. Subjects in this study will be taking approximately 24 teaspoons daily. The primary benefit derived from this study will be the knowledge as to whether FOS can enhance absorption of the soy in postmenopausal women by increasing the friendly bacteria in the bowel. If this is the case, a larger trial will be designed, and ultimately medical nutrition recommendations for the addition of FOS to one's daily soy regimen may be published in the scientific literature.

7. Compare the risks versus the benefits.

The risks for participating in this study are minimal. The soy and FOS products are both available commercially. The subjects enrolled in this study will be healthy with no chronic diseases. The knowledge gained from this study will provide medical and nutrition information regarding soy and FOS and may ultimately contribute to published recommendations pertaining to the inclusion of FOS in the diet when taking soy.

8. Will the subjects for the study be paid for participating in this study? Yes X No □

If yes, how much? $200

Will subjects be paid for selected activities (e.g., blood drawing) or for general participation in the study?

NOTE: All information concerning payments, including the amount and schedule of payment, must be included in the consent form.

Subjects will be paid $100 following each GCRC admission. This includes the completion of the diet and gastrointestinal sheets recorded by the subject four days prior to each of the two (2) GCRC admissions.

Is there any other inducement? Yes X No □ If so, please describe.

Subjects/significant others will also receive payment for any parking expenses for each GCRC admission.

9. Will advertising be used to recruit subjects? Yes X No □ If yes, attach a copy of the proposed advertisement.

Please see attached. The advertisements will be posted in the participating gynecologist’s waiting rooms (the currently participating Dr. Kathleen Lutter, MD, and the new participating gynecologists, Dr. Beth Boyles, MD, and Dr. Donna T. Diaz, MD,) and the Ohio State University and OSU Hospital.

SOURCE OF FUNDING FOR PROPOSED RESEARCH: (Check A or B)

A. OSURF: Sponsor □ RF Proposal/Project No. □

B. Other (Identify) □ Partial: Abbott Laboratories/Ross Products Division

Information about the funding/sponsorship of human subjects research activities is required for administrative purposes. Such information is generally not required as part of the human subjects review process.
APPENDIX B

GCRC APPLICATION FORM
GCRC Protocol Application-FINAL for submission

Protocol ID: (Administrative use only)

A. Title: The Effect of Fructooligosaccharides (FOS) on the Absorption of Isoflavones from Soy Protein in Postmenopausal Women- A Pilot Study

B. Principal Investigator

Name and degree: Dr. Anne M. Smith, PhD, RD, LD
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e-mail: smith.23@osu.edu

Completed OSU Human Subjects’ Protection Training: Yes X No ___

Co-Investigators (duplicate as needed or eliminate if none)

Name and degree: Maureen E. Geraghty, MS, RD, LD
Social Security Number: 273-64-9733
Academic Title: Graduate Research Associate, Doctoral Student
College: Human Ecology
Department: Human Nutrition
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Completed OSU Human Subjects’ Protection Training: Yes X No ___

Study Coordinator (all information same as above)

Name and degree: Maureen E. Geraghty, MS, RD, LD
Academic Title: 
College: 
Department: 
Division: 
Address: 
Phone: 
FAX: 
Pager/cellular phone: 
e-mail: 
Completed OSU Human Subjects’ Protection Training: Yes X No ___
C. Specific Aims

Primary Objective and Response Variable:

The specific aim of this Pilot Study is to determine whether the short term addition of FOS (9-10 g/d for 3 weeks) to the diet of postmenopausal women consuming 26 g soy protein/d (80 mg isoflavones) will enhance the absorption of the isoflavones as measured by urine isoflavones and plasma isoflavones.

Secondary Questions and Response Variables:

As this is a pilot study, several response variables are being explored. Those outcomes with statistical and/or clinical significance can subsequently be studied in a larger prospective trial.

- To examine what changes, if any, may occur in the gut microflora following SOY intake and SOY + FOS intake treatment periods.
- To assess β-glucosidase activity in the fecal samples following SOY intake and SOY + FOS intake treatment periods.
- To determine if a correlation exists between breath hydrogen production and microbial populations found in the fecal samples.
- To evaluate whether plasma selenium and selenoprotein levels change following SOY intake and SOY + FOS intake treatment periods.
- To ascertain the subjects’ compliance (adherence) to the study intake guidelines for daily protein (soy and non-soy protein) and to determine the effect of soy protein intake on daily protein intake by comparing (through diet records) total protein intake during the baseline and washout periods with protein intakes during interventions 1 & 2.
- To measure gastrointestinal tolerance during the SOY intake and SOY + FOS intake treatment periods by using a previously published Gastrointestinal Symptoms Scale.

D. Background & Significance

As women approach mid-life, they continually seek new ways to address a variety of the accompanying health problems, including, 1) an increased risk and incidence of coronary heart disease (CHD), 2) perimenopausal symptoms such as hot flashes; and 3) decreased bone mineral density and possible osteoporosis. Women in this century will live one-third to one-half of their lives after menopause, and many either cannot or will not comply with conventional therapy, such as hormone replacement. Many women have become proactive in researching their options for optimal health care, especially in context of the early termination of the Women’s Health Initiative trial which addressed the long-term effects of hormone replacement therapy (1). The addition of soy to the diet is a modality recommended by some health care professionals to prevent disease or reduce the severity of symptoms associated with menopause such as hot flashes. Soy products are safer than the majority of herbal therapies available to postmenopausal women.

Many studies have been conducted regarding the effect of soy products on health problems of peri and postmenopausal women. In a meta-analysis of 38 clinical trials (with both women and men) (2), a complete regression analysis was performed using different levels of soy protein in the diet. The model predicted that a daily intake of 26 grams of soy protein would be associated with an 8.9 mg/dl change in
total serum cholesterol, and that a 50 gram soy protein intake would be correlated with a serum cholesterol decrease of 17.4 mg/dl. The results of a more recent study suggest that the isoflavones in dietary soy impart a protective role against cardiovascular disease (CVD) in postmenopausal women (3). Women with a high usual isoflavone (specifically genistein) intake had a significantly lower body mass index (BMI), waist-circumference, and fasting insulin than those with negligible intakes. An adjusted analysis revealed that isoflavone intake was positively associated with HDL cholesterol and inversely related to a 2-h post challenge insulin following a 75-g oral glucose tolerance test.

The role of soy in alleviating peri-menopausal symptoms (specifically hot flashes) other than high serum lipids is less clearly defined. Washburn et al (4) tested the daily provision of 20 grams of isolated soy protein with isoflavones to peri- and postmenopausal women in either one dose or two equal doses. Compared to placebo, the twice daily doses significantly reduced severity of hot flashes (P<0.004). In a similar study, Murkies et al (4) found no significant differences between groups of menopausal women, consuming soy flour or wheat flour; both groups reporting a decrease in the frequency of hot flashes.

The positive effect of soy isoflavones on the reduction of bone loss in several animal models has been reported (6-9). One of the first human clinical trials addressing this benefit was published in 1998 by Potter et al (10). Their premise, that since isoflavones possess biochemical actions similar to estrogens, isoflavones may also influence several biological processes, particularly lipid and bone metabolism. Sixty-six women were randomly assigned to one of three protein interventions. Two groups of women were provided with 40 g/d of soy protein which contained either 56 mg. or 90 mg. of isoflavones. The third group was given 40 g casein (the isoflavone-free group). The three groups consumed their randomly assigned protein mixtures in addition to a National Cholesterol Education Program (NCEP) Step I diet for a period of six months. There was an increase in high-density lipoprotein (HDL) cholesterol starting at week 7 of the group ingesting the 90 mg/d of isoflavones, but the increased HDL did not appear until week 18 in the group ingesting 90 mg of isoflavones per day (P > 0.05). Non-HDL cholesterol decreased at week 24 (P < 0.05) in both soy protein groups compared to the casein group. However, it was 90 mg/day of isoflavones which had a significant effect on bone mineral density. In addition to the delayed decrease in bone density compared to the control (casein diet) after six months, ingestion of the 90 mg/d isoflavone slightly increased bone mineral content of the lumbar spine (transversal bone (T) < 0.05).

Thus, the moderate level of isoflavones was more effective in terms the lipid profile benefits, but it was the higher level of isoflavones that had the positive effect on bone mineral content. Overall, the authors concluded that although many questions about the use of soy in menopausal women have yet to be answered, the dietary inclusion of isoflavones-containing soy products may provide an efficacious form of alternative therapy for the enhancement of health in postmenopausal women (10).

The results of this study led health care practitioners to question whether it would be beneficial to recommend the lower or the higher levels of isoflavones to postmenopausal women for maximum benefit for their cardiovascular and bone health. Many of the soy protein powders currently on the market contain approximately 55 mg. of isoflavones per 20 grams of protein, necessitating the consumption of closer to 40 grams of protein (as in the Potter study) to achieve the bone-sparing effect of the soy. According to Dr. John Erdman, even though the women in this study were counseled to reduce their non-soy daily protein intake, (since they were already receiving 40 grams/d of soy or casein protein), many did NOT do so (11) which could lead to weight gain or to renal implications resulting from consumption of excessive protein in predisposed populations.

Soy, a phytoestrogen, has a biochemical structure very similar to estrogens (12), and selenium levels have been associated with estrogen metabolism (13). The risks of disturbances in trace mineral nutrition and metabolism are substantial following menopause (14). In a cross-sectional, three generational study addressing the selenium status of women, Smith et al (15) found that the grandmother group (postmenopausal women) had the lowest plasma selenium levels compared to daughters (19-29 yr) and mothers (40-58 yr). A positive correlation between plasma estrogen and plasma selenium values in all three groups was reported (15). No study has yet been conducted where selenium levels are measured in postmenopausal women following soy administration.
The bioavailability of isoflavones is also critical to consider. The isoflavone, genistein, is a glycoside conjugate which most likely requires a glycosidase in order to be converted to an aglycone derivative (or mammalian metabolite) before it can be available for intestinal absorption (e.g., genistin → genistein or dihydrogenistein). Recently, it was reported that short term (7 day) feeding of fructooligosaccharides (FOS) enhanced the absorption of isoflavones, specifically genistin and daidzein (16, 17). It was speculated that the mechanism involved the fact that FOS is a known bifidogenic factor, and bifidobacteria have glycosidase activity. The effects of FOS on isoflavone absorption in humans, however, are unknown. Studies in humans are needed to corroborate these results. It has been recently shown in healthy humans, however, that isoflavone glycosides (e.g., genistin and daidzin) are not absorbed intact across the enterocyte (FOS was not administered in this study), and that the presence of β-glucosidases is required for hydrolysis of the β-glycosides for peripheral circulatory uptake (18). If FOS can enhance isoflavone absorption, it stands to reason that less soy protein would need to be consumed to achieve the beneficial effects associated with these phytoestrogens. FOS is also associated with many health benefits not imparted by soy supplements alone, such as digestive tract health (19), reduced serum lipid levels (20), the enhanced absorption of minerals (21), and anticarcinogenic mechanisms (22). The study of the effects of diet in the form of prebiotics on the manipulation of the microbial community is considered critical by prominent researchers in the field (23) and is currently experiencing high profile coverage at major biological sessions (24).

E. Preliminary Studies [If this is a pilot study, this section is not applicable.]

This study is indeed a pilot study.

F. Experimental Design & Methods

Study Design:

This is a prospective, single-blinded, placebo-controlled parallel clinical pilot study with a randomized block design. Subjects will act as their own control. This study will be single-blinded by providing a powder as a placebo to the FOS. When subjects are assigned Treatment A, they will receive Soy and the placebo powder, and when they are assigned Treatment B, subjects will receive Soy and the FOS powder.

Inclusion and Exclusion criteria for study participants:

Twenty-four (24) healthy postmenopausal women will be recruited for this pilot study. The following criteria will be used for subject eligibility: 1) Subject has not taken any medication known to alter lipid, bone, or calcium metabolism including hormone replacement therapy, or any medication affecting nutrient metabolism or absorption or over the past 6 months; 2) Subject has not had a menstrual period within the 12 months before the study is initiated but is not older than 70 years old; 3) Subject has no history of gastrointestinal or malabsorptive disorders (Crohn's disease, ulcerative colitis, gluten enteropathy, irritable bowel syndrome, short bowel syndrome secondary to surgery, etc.); or metabolic diseases; 4) Subject has no food allergies; 5) Subject has not taken soy products for the past three months and is not currently taking any type of soy and or isoflavone supplements; 6) Subject has not taken fructooligosaccharides supplements for the past three months and is not currently taking fructooligosaccharides supplements; 7) Subject agrees to maintain the same dosage of any mineral or vitamin supplements they were consuming until completion of the study (if subject is taking herbal supplements, they will agree to discontinue use until the end of the study); 8) Subject has not taken antibiotics or probiotics for the past 3 months; 9) Subject is willing to be admitted to the GCRC two (2) separate times, for approximately 20 hours each time over 5 consecutive weeks to obtain: a) urine samples for urine isoflavones; b) breath hydrogen samples; c) fecal samples for molecular characterization of the intestinal microflora; d) four (4) blood draws over 5 weeks for plasma selenium; and four (4) blood draws for plasma isoflavones; and 10) Subject is willing to complete food records and gastrointestinal tolerance scales during two of the five weeks of the study.

Subjects will be counseled to eliminate foods inherently high in soy and FOS (other than the soy and FOS...
intervention supplements) from their diet. (Please see form following Bibliography, entitled, "Guidelines for following a Soy-free and FOS-free diet")

**Method(s) of Subject Evaluation:**
Subjects will be evaluated in terms of several outcome measures:

- Urine isoflavone concentrations
- Breath Hydrogen
- Microfloral characterization of fecal samples via molecular techniques
- \( \beta \)-glucosidase concentrations of the fecal samples
- Plasma selenium levels
- Plasma isoflavone levels
- Patient records: a) Diet records; and b) Gastrointestinal tolerance scales

**Urine Isoflavones**

Twenty-four hour (24-h) urine collections will be obtained from the subjects at four (4) time points during their inpatient stays at the General Clinical Research Center during this 11-week study. Subjects will begin collecting urine on the Thursday of each admission at 6:00 a.m. until 6:00 a.m. the following morning. Subjects will use a simple portable device (Urine Mate P, Sumitomo Baeelite, Tokyo) for proportional sampling. Vitamin C (10 g/L) and NaH\(_2\) (0.003 mol/L final concentration) will be added to the storage bottles to prevent isoflavone oxidation and bacterial contamination (15). Storage bottles will be labeled with the subjects' code numbers and collection dates. Samples will be frozen in the storage bottles at -80 until assayed. Validated methods for assaying the isoflavones (daidzein, genistein, and glycitein) and the daidzein metabolites, 6- desmethylangolensin (O-DMA) and equol, and the genistein metabolites, 6-O- DMA and dihydrogenstein in urine using high-performance liquid chromatography (HPLC) have recently been described (25,26).

**Breath Hydrogen**

Interval samples of end-expiratory hydrogen will be collected in accordance with proper technique (27). Prior to the study the co-investigator will train Nursing as to the proper breath hydrogen sample collection technique so that they will be able to provide the appropriate teaching for the study subjects.

These samples will be collected from the mouth of each subject in 80 mL plastic syringes fitted with a stopcock after a 12 hour overnight fast during each stay in the GCRC. Sampling will begin at 5:45 a.m. and last until 2:00 p.m. and samples will be taken at the following intervals: -15 minutes, 0-time, 2, 4, 6, and 8 hours. The collection syringes will be labeled with the subjects' code numbers and collection dates. Hydrogen concentrations will be measured on a compact gas chromatograph microalyzer (Quintron Instrument Company, Inc., Milwaukee).

A standard meal will be provided at 11:00 a.m., five hours into the testing. This meal (One 237 mL can of Ensure® Plus) is known to generate negligible hydrogen during digestion and metabolism. A full meal will be provided when the breath hydrogen test is over at 2:00 pm.

**Microfloral characterization of fecal samples**

Subjects will collect one stool sample during each stay at the GCRCG. (Subjects have all day Wednesday before that same Wednesday evening GCRC admission until discharge on the Thursday afternoon during the approximately 20-hour admission. Should the subject be unable to produce a stool, the co-investigator will arrange for pick-up of a stool over the weekend after discharge. The sample will be placed in a sterile cup with minimal headspace, and immediately placed on ice. A microbiology student will board the sample and to prepare it for microbiological plates within 24 hours. Before preparation of the stool for microbiological plating, 1 gram of sample will be obtained from the center of the stool (28) and placed into 2 cryovials (each to hold 0.5 g of sample and frozen at -70 °C). The sterile cups and cryovials will be...
labeled with the subjects' code numbers and collection dates. The vials will be later thawed for DNA extraction and subsequent PCR-DGGE (Polymerase Chain Reaction- Denaturing Gradient Gel Electrophoresis) analysis (28,30).

\(\text{L-glucosidase concentrations of fecal samples}\)

Beta-glucosidase (\(\text{(L-glucosidase)}\)) activity will be measured to assess microbial metabolic activity. Before preparation of the stool for microbiological plating, another 1 gram sample will be obtained from the center of the stool and placed in cryovials and frozen at -70° C. The vials will be labeled with the subjects' code numbers and collection dates. The vials will be later thawed and fecal suspensions will be prepared by taking 1 g of homogenized fecal material and suspending it in 9 mL of phosphate buffer (50mM, pH=7). A handheld homogenizer will be used to homogenize samples for 2 min. followed by a 10 minute treatment with a sonic dismembrator. Centrifugation will be employed to remove particulate matter (9500 g, 10 min) and the supernatant will be used to determine enzyme activity. The hydrolysis reaction will be conducted using 4-methyl umbelliferyl \(\beta\)-D-glucosidase (Sigma, St Louis) at 37°C for 5 min. The reaction will be arrested by transferring 200 mL of reaction mixture into 3.8 mL of sodium borate solution (0.2 M, pH 10.5). Fluorescence will be measured and standard curves prepared. Enzyme activity will be defined as the amount of methyl umbelliferone (\(\mu\)M) released per minute per gram of dry weight of fecal matter (29).

\(\text{Plasma samples}\)

Blood will be drawn for both plasma isoflavones and for plasma selenium samples on the Thursday of each of the two (2) GCRC admissions.

For plasma isoflavones, 3 mL blood samples will be drawn at 2 and 8 hours following ingestion of a soy product with known levels of isoflavones. Samples will be drawn into evacuated tubes containing potassium EDTA. They will be labeled with the subjects' code numbers and collection dates. They will be immediately placed on ice and then centrifuged (10 min, 4°C, 3000 X g) to separate the plasma and then will be stored at - 80°C until analysis (26).

Five (5) mL of blood will also be drawn at the same times as the plasma isoflavones - at 8:00 a.m. and 2:00 p.m. The blood for the selenium samples will be drawn into evacuated tubes with potassium EDTA, immediately placed on ice, and centrifuged at 1400 rpm in a refrigerated centrifuge for 20 minutes to separate plasma from red blood cells (RBCs). Using a Pasteur (transfer) pipette, the plasma will need to be transferred into three of the provided microcentrifuge tubes. No RBC should be inadvertently mixed with the plasma samples. Tubes will be labeled with subject's code number and collection date. Samples will be analyzed for total selenium, glutathione peroxidase (GPX) and Selenoprotein P.

\(\text{Subject Records}\)

Subjects will keep diet intake records for four (4) days of each of the two (2) weeks preceding the GCRC admissions. It has been established that four days of dietary intake (2 weekdays and both weekend days) provides an accurate representation of the subject's intake for that week (31).

Gastrointestinal Tolerance Records will be completed the same four days as the diet records. The subjects will be instructed to self-report symptoms on an hourly basis using a record sheet (32). The degree to which they may experience the various symptoms (bloating, flatulence, abdominal pain, diarrhea/loose stools, headache) will be indicated by placing an "X" on a 6 cm line numbered 0 to 6. The symptom severity will have a corresponding number for quantification and statistical purposes: 0 = none, 1 = slight, 2 = mild, 3 = moderate, 4 = moderately severe, and 5 = severe (32,33).

\(\text{Treatment Schedule}:\)
The duration of this study for each of the twenty-four (24) subjects is five weeks. It will be attempted to enroll four of the subjects each week, with subjects being enrolled for six consecutive weeks as schedules (GCRC and patient) permit. The study is comprised of two periods. The first period is the Baseline Period with a duration of two (2) weeks. The second period is the Treatment Period, with a duration of three (3) weeks. Subjects are randomized to either Treatment A - the "SOY only" treatment or Treatment B - the SOY + FOS treatment.

Subjects will be counseled to eliminate foods inherently high in soy or FOS (other than the soy and FOS intervention supplements) from their diet for the duration of the 5 week study. During the Baseline Period, subjects will eat their normal diet with the exception of the aforementioned foods high in soy or FOS. For the Treatment Period, subjects will receive instruction as to how to keep protein intake similar to baseline during the treatment period.

If the subjects are randomized to Treatment A, they will prepare a Health Source © Soy Protein Shake (Ross Products Division, Abbott Laboratories, Columbus, Ohio) every morning with 2 scoops of powder and 8 fl oz of skim milk. A 2-scoop serving provides 26 g protein and 80 mg isoflavones. They will also receive a placebo powder (dextrose) to be dosed like the fructooligosaccharides (FOS).

If the subjects are randomized to Treatment B, they will receive the Health Source © Soy Protein Shake and the Nutraflora® Short Chain Fructooligosaccharide FOS Powder (FP5), Golden, Colorado. GTC Nutrition LLC. The FOS powder will be dosed incrementally to a total of 10 g/day by day the fourth day of the intervention period, and subjects will be instructed to take three (3) evenly divided doses to enhance tolerance.

GCRC Subject Visits
A screening visit will be scheduled once subjects are identified by their physician to determine eligibility. If eligible, subjects will be admitted to the GCRC two (2) times during the five (5) week study, during the last week of each study period (for Baseline, this is week 2 of the study; for the Treatment Period, this is week 5 of the study.) They will be admitted on a Wednesday evening before 6 pm and will be discharged from the GCRC on the next day (Thursday) at 2:00 pm. (Admissions depicted by an ▼ below):

Baseline Period ▼  Treatment Period ▼
(Weeks 1-2) (Weeks 3-5)

During the week prior to each of the two admissions, subjects will complete four days of dietary intake records (two weekdays and two weekend days) and will complete Gastrointestinal Tolerance Scales on the same four days.

The other measurements will be taken at the specified visits as follows:

Baseline Period Visit - Sample collections and Meals
(Week 2)

Subjects:

- are admitted to the GCRC by 6:00 pm the Wednesday of the designated week. Height and weight are recorded at Baseline.
- fast from 6:00 p.m. Wednesday evening to 6 a.m. Thursday morning.
- will participate in one breath hydrogen collection at 6:00 am (since this is a non-treatment period).
- will provide blood samples at 8 am and 2 pm.
- will provide 24 hour urine collections between 6:00 am Thursday morning and 2:00 pm Thursday. The co-investigator will pick up the remainder of the 24-h urine collection (from 2:00 pm Thursday to 6:00 a.m. Friday) from the patient's home.
- will provide a small amount (approximately 20 g) of stool between 6:00 p.m. Thursday morning and 2:00 pm Thursday afternoon. The co-investigator can pick up a sample later if patient is unable to provide one on the GCRC.
will be fed a meal (regular diet-free of soy and FOS) at breakfast and lunch on Thursday.

Treatment Period (Week 5)

Subjects:

- are admitted to the GCRC by 6:00 p.m. the Wednesday of the 5th week. Weights are recorded.
- fast from 6:00 pm Wednesday evening to 6 am Thursday morning.
- will participate in breath hydrogen testing at 5:45 am, 6:00 am, 8:00 am, 10:00 am, 12 noon, and 2:00 pm.
- will provide blood samples at 8 am and 2 pm.
  - will provide a 24-hour urine samples between 8 am Thursday morning and 2 p.m. Thursday afternoon. The co-investigator will pick up the remainder of the 24-h urine collection (from 2:00 pm Thursday to 8:00 a.m. Friday) from the patient's home.
- will provide a small amount (approximately 20 g) of stool between 6:00 am and 2:00 p.m. Thursday.
- will be given the Treatment A or Treatment B (whichever one to which they are randomized) at 6:00 a.m. Thursday morning.
- During the breath hydrogen sampling on Thursday, subjects will be provided with one can of Ensure Plus at 11:00 am, and they will be served a regular meal following the final breath hydrogen test at 2 pm.

Physician Coverage

Dr. Steven Clinton has been contacted and invited to be the study physician. He has suggested that his new colleague, Assistant Professor, Dr. J. Paul Monk, M.D., act as study physician, and that he (Dr. Clinton) will provide backup when necessary in the event Dr. Monk is out of town.

Biological Specimen Collection and Processing:
(Please also see Methods of Subject Evaluation)

Urine samples:

Twenty-four hour urines will be collected by the subjects during each of the two visits. About the sample Samed. am, and when the 24-hour collection is completed, the co-investigator will pick up the samples, place them on ice, prepare the samples, and store them at -80°C in storage bottles in the Dept. of Human Nutrition. Specimen labels will be provided by the GCRC.

Breath Hydrogen samples:

Breath Hydrogen samples will be collected in 60 ml syringes provided by the co-investigator. Once collected sample will be placed in the GCRC freezer. They will be picked up by the co-investigator the same day as sample collection and transported to the Dept. of Human Nutrition and frozen there at -20°C. Specimen labels will be provided by the co-investigator.

Fecal Samples:

Subjects will provide only one stool sample in the 48 hours between the Wednesday (day the evening GCRC admission) and the Friday morning of discharge. The investigator will provide the stool collection container, which ideally will have minimal headspace (e.g. small containers). Once the stool is collected, it will be refrigerated or placed on ice, and a microbiology student will be paged for immediate sample pick.
up, for subsequent processing, and transfer into cryovials and for freezing at -70°C. Specimen labels will be provided by the GCRC.

**Plasma samples:**
Blood drawn from subjects will be immediately placed on ice and centrifuged by the Core Lab at the GCRC and frozen at -70°C. Tubes in which to store and freeze the sample after centrifugation will be provided by the investigator. Specimen labels will be provided by the GCRC.

**Subject Records:**
Subject records (diet records and gastrointestinal tolerance records) will be collected and reviewed during each admission by the co-investigator. She will enter all data onto the ESHA nutrient analysis software program (ESHA Research, Salem, OR).

**Data Collection and Management Plan:**
The co-investigator will develop all case report forms (data forms) and will manage all data. Subjects' samples will be analyzed by the co-investigator and entered into Microsoft Excel® by this same investigator.

**Biostatistical Design and Analysis:**

**Sample Size Justification:**
The statistical program “nQuery Advisor” Version 5 was used to carry out the power analysis to justify the sample size. A sample size of 12 in each group will have 83% power to detect an effect size of 1.250 using a two sample independent t-test with a 0.050 two-sided significance level. Likewise, a sample size of 11 in each group will have 79% power to detect an effect size of 1.250 using a two sample independent t-test with a 0.050 two-sided significance level. Although this study has not yet been conducted in humans, the “Effect size” of 1.250 is reasonable based on changes seen in animal studies where soy and FOS were administered (16,17).

**Analysis Plan:**
The primary response variable is the change in soy isoflavone absorption that may occur from the provision of H-U on the diet. This will be measured by plasma and urine isoflavone concentrations. For each of these variables, the mean changes for the Placebo Group (A) and the Treatment Group (B) will be compared using a 2 Independent sample t-test assuming the changes are normally distributed. Equality of variances will be checked and an appropriate t-test will be performed. If normality fails to be demonstrated, the Wilcoxon test, the nonparametric equivalent of the t-test, will be used for comparison. The level of significance will be set at 0.05.

The other response variables are as follows and changes will be reported with descriptive statistics (calculations will include the means, standard errors, confidence intervals and testing for relevant effects and correlations: 1) gut microflora (numbers); 2) β-glucosidase activity; 3) breath hydrogen; 4) plasma isoflavones; and 5) plasma selenium. Subjects’ diet records will be analyzed to assess compliance with study guidelines for daily protein (soy and non-soy) intakes. Gastrointestinal Tolerance records will be comprised of symptoms with a numeric scale that increases with the intensity of the perceived symptom, (e.g. a rating of “5” for bloating is worse than a rating of “2”). Differences in symptoms will be evaluated for the two treatments.

**Dropouts**
The block randomization design (four subjects in the first batch going through the GCRC- two on Treatment A and two on Treatment B, and so on for the next 6 batches of four) will allow us to observe any attrition that may occur and enroll the appropriate number to achieve a total of twenty-four (24) subjects.

**Technical Support**
The co-investigator has requested that Dr. Raj Nagaraja, PhD supervise her statistical work. He has acquainted her with the “JMP IN® software by SAS and the corresponding primer, “A Guide to Statistics GCRC revised application (06-26-03)-Parallel design page 9”
and Data Analysis Using JMP® and JMP IN® Software® (by Sall, J., Lehman A., Creighton L., Duxbury Thomson Learning, 2001). The co-investigator will be using the 4th edition for her analyses.

G. Subject Selection/Female & Minority Recruitment

National distribution of the disease (percent). Please note: The population being studied is healthy postmenopausal women.

Source: U.S. Census Bureau, Current Population Survey, March 2000, (healthy women ages 45-64)

<table>
<thead>
<tr>
<th>Gender</th>
<th>Race</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>American Indian or Alaskan Native</td>
<td>Asian &amp; Pacific Islander*</td>
</tr>
<tr>
<td></td>
<td>Black or African American</td>
<td>Native Hawaiian or Other Pacific Islander*</td>
</tr>
<tr>
<td>Female</td>
<td>0.845</td>
<td>3.668</td>
</tr>
<tr>
<td>Male**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Local distribution of the disease (percent)

Source: U.S. Census Bureau- Franklin County OH, (healthy women ages 45-64)

<table>
<thead>
<tr>
<th>Gender</th>
<th>Race</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>American Indian or Alaskan Native</td>
<td>Asian &amp; Pacific Islander*</td>
</tr>
<tr>
<td></td>
<td>Black or African American</td>
<td>Native Hawaiian or Other Pacific Islander*</td>
</tr>
<tr>
<td>Female</td>
<td>0.3</td>
<td>3.1</td>
</tr>
<tr>
<td>Male**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Actual planned recruitment (numbers).
(Calculations derived from local distribution above) Investigator would welcome women from all races/ethnicities.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Race</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>American Indian or Alaskan Native</td>
<td>Asian &amp; Pacific Islander*</td>
</tr>
<tr>
<td></td>
<td>Black or African American</td>
<td>Native Hawaiian or Other Pacific Islander*</td>
</tr>
<tr>
<td>Female</td>
<td>0.072</td>
<td>0.744</td>
</tr>
<tr>
<td>Male**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

* The Census Bureau data cited here combines the Categories “Asian” and “Pacific Islander.”
** The study’s objective is to study the effects of FOS and SOY in postmenopausal women only.
H. Human Subjects

Twenty-four (24) menopausal women will be recruited for this pilot study. The investigators have submitted the appropriate forms to the Ohio State University Biomedical Institutional Review Board (IRB) where the protocol and informed consent form will be evaluated independently.

Sources of research material

Specimens from the subjects will be obtained prospectively in the form of blood samples, urine collections, stool collections, breath hydrogen collections, and written records (diet record and gastrointestinal tolerance records). All specimens will be used for research purposes only. The subject's medical records will be reviewed during screening to determine subject eligibility. Confidentiality will be maintained by using subject initial and numbers in place of full names. All screening and other study information/data will be kept in a locked file drawer in the co-investigator's office at all times when not in use.

Informed Consent (Describe who will be responsible for initiating the consent process, as well as how and where the study participants will be consented. For study participants ≤ 18 years old consent/assent to participate must be assured per OSU ORRP-IRB Policy. [NOTE: Copies of the signed consent(s) (and assents, as applicable) for every study participant will be maintained as part of a permanent record; for inpatient studies, a copy of the signed written consent document will be maintained in the appropriate hospital medical record; for outpatient studies, the signed written consent document will be maintained in a study file on the GCRC. A copy of each version of the written informed consent document approved by the OSU ORRP-IRB must be forwarded to the GCRC, in order to ensure compliance to the principles of the process of consent].

Potential eligible subjects will meet with the principal investigator and/or co-investigator in the subject's gynecologists' private meeting room where all questions will be answered and informed consent obtained. Copies of the signed consents for every study participant will be maintained as part of a permanent record, in this case the study file in the GCRC. A copy of each version of the written informed consent document approved by the OSU ORRP-IRB will be forwarded to the GCRC, in order to ensure compliance to the principles of the process of consent.

(Co-investigators note) The following templates have been added to the written informed consent document - MEG. (NOTE: It is recommended that when possible these sections be included in the initial OSU ORRP-IRB submission):

(Confidentiality section) Since a portion of this research project will be conducted at The Ohio State University (OSU) General Clinical Research Center, the Programs/Medical Director, Research Subject Advocate and/or member(s) of the nursing staff, may review my medical records. A copy of this written informed consent document will be included in my General Clinical Research Center medical record. A copy has been given to me for my own records and reference.

(Contact section) Should I have any questions regarding my participation associated with the study activities on the OSU General Clinical Research Center (GCRC), I may contact the Research Subject Advocate for the OSU GCRC at (614)293-9274.

If the protocol will include any overnight stay(s) on the GCRC, then that must be included in the written informed consent document.

A copy of the effective signed informed consent for each study participant must be submitted to the GCRC for the respective protocol/study participant file prior to the initiation of any protocol procedures to be conducted.
11. Personal communication with Dr. John W Erdman, Jr., March 3, 2000
26. Richelle, M. Pridmore-Marten S, Bodenstab et al: Hydrolysis of isoflavone glycosides to aglycones by β-
glycosidase does not alter plasma and urine isoflavone pharmacokinetics in postmenopausal women. J Nutr
2002;132:2587.
27. Runesson JI, Hamberg O, Gudmund-Hoyer E: Interval sampling of end-expiratory hydrogen (H2)
28. Boddington RK, Williams CH, Chen S: Dietary supplement of isosugar alters the fecal flora and decreases
29. Simpson JM, Martineau, Jones WE et al: Characterization of Fecal Bacterial Populations in Canines:
30. QiAamp® DNA Stool Mini Kit Handbook – For DNA purification from stool samples. Product #51504,
32. Lin M-Y, Dipalma JA, Martini MC et al: Comparative effects of exogenous lactase (β-galactosidase)
Guidelines for following a
"Soy-free" and "FOS-free" diet

If you are considering enrolling in this study which is looking at the effects of a special fiber (FOS) and how it may help soy to be better absorbed from your digestive tract to your blood), it's important that your diet now and in the past three months meet the following criteria:

1) You have not taken any soy products in the past three (3) months;
2) You are not currently including soy products in your diet; and
3) You have not taken any FOS (Fructooligosaccharide) supplements over the past three months; and finally,
4) You are not currently taking FOS supplements.

The lists are helpful in determining whether you have had soy-containing foods or FOS containing foods in your diet over the past year. We are also asking you to continue to eliminate those from your diet at this time and during the study:

**Soy Products**
- Soy milk
- Soy Protein Isolate (found in many Soy Powders)
- Soy Bars
  (Brand names such as Genisoym™ Health Source™ are often found on bars and powders)
- Meat alternatives, such as TSP- (Textured Soy Protein) or TVP-(Textured Vegetable Protein)
  These are found in veggie burgers, veggie chili, veggie sausages, etc. Worthington Foods is a local distributor of vegetarian meat alternative products.
- Soy nut butter
- Whole Soybeans, roasted or broiled
- Miso
- Tofu
- Tempeh
- Soy flour
- Textured Vegetable Protein

**FOS**
- Prebiotic supplements only (those with fructooligosaccharides).

*It is nearly impossible to get enough FOS in foods to affect the study, so no foods are listed to be avoided. We recommend that you do avoid Probiotic supplements and cultured yogurt/dairy products- those with live lactobacillus and other probiotic cultures during the study.*
GCRC PROTOCOL RESOURCE REQUEST

I. JUSTIFICATION FOR USE OF THE FACILITY

The requisite fasting (6 p.m. - 8 a.m.) followed by 8 hours of testing (multiple blood draws and breath hydrogen samples) render it necessary to have subjects medically monitored and to have sequential samplings performed by trained professionals with appropriate supplies and universal precautions. Compliance with the number and types and exact methods of testing will also be greatly improved in the GCRC setting.

II. STUDY TYPE

<table>
<thead>
<tr>
<th>CHECK ONE</th>
<th>HOSPITAL LABS R# (for labs billed to your grant)</th>
<th>OSURF ACCOUNT # TO BILL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Funded Study (non-industry)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Un-funded Study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Industry-Sponsored Research (partially- only $4,000 to cover only soy powder made by the company (Ross Products) and some of the kits for sample analyses by co-investigator. Please note that this is not an Industry driven protocol; the co-investigator approached industry with the idea and a request for product and money for analysis kits. This is independent work for the co-investigator’s doctoral dissertation.</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

Is this a pilot study? _Yes_ ☑ _No___
Is this study a multi-center trial? _Yes__ ☑ _No_ ☑
Is this study a clinical trial? _Yes__ ☑ _No_ ☑

Phase: __I__ __II__ __III__ __IV__

Please list funding source(s): 1) Ross Products Division- Soy product & $4,000 for analytical kits. 2) GTC Nutrition LLC - Fructooligosaccharide Powder.

Please list IRB protocol number: Pending
III. PROPOSED STUDY DATES (from initiation to close of study on the GCRC):

Start Date: 07/28/03
  MM/DD/YYYY

Stop Date: 12/12/03
  MM/DD/YYYY
IV. PROJECTED UTILIZATION

A. Total Utilization for the Lifetime of the Protocol

<table>
<thead>
<tr>
<th>INPATIENT DAYS</th>
<th>OUTPATIENT VISITS</th>
<th>SCATTER DAYS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Patients</td>
<td>Total Patients</td>
<td>Total Patients</td>
</tr>
<tr>
<td>Number of Days per patient</td>
<td>Number of Visits per patient</td>
<td>Number of Days per Patient</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

B. First Year Utilization through November 30 of the current calendar year (The study would not start until 2003)

<table>
<thead>
<tr>
<th>INPATIENT DAYS</th>
<th>OUTPATIENT VISITS</th>
<th>SCATTER DAYS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Patients</td>
<td>Total Patients</td>
<td>Total Patients</td>
</tr>
<tr>
<td>Number of Days per patient</td>
<td>Number of Visits per patient</td>
<td>Number of Days per Patient</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Scatter Days are for investigations in which the study participants' medical condition requires hospitalization in an acute care setting while on a research protocol. The GCRC will cover room charges if the study participant is occupying that bed solely for research purposes. Please contact the GCRC Administrative Director (293-4406) for additional clarification.

V. SPECIALIZED NURSING REQUEST (check all that will be required)
Please contact the GCRC Director of Nursing (614-293-9749) with any questions before filling out this section.

- Administer Questionnaires
- Administer Study Medication
- EKG
- Phlebotomy
- PKs or Frequent Blood Sampling X
- Telemetry
- Scatter Nurse
- Teaching, specify type: Breath Hydrogen Technique X
- Other: Long term nutrition and health benefits of soy and of fructooligosaccharides X

VI. INFORMATICS REQUEST
None, thank you.

- Hospital Laboratory Data Download
- Core Lab Data Download
- Develop Scannable/FAXable Forms (Teleform)
- Database Application Development
- Use of the Computer Lab
- Website Development
VII. BIONUTRITION REQUEST

<table>
<thead>
<tr>
<th>Meals:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular</td>
<td>X</td>
</tr>
<tr>
<td>Specialized (i.e., diabetic)</td>
<td></td>
</tr>
<tr>
<td>Controlled nutrient:</td>
<td></td>
</tr>
<tr>
<td>• Soy shake</td>
<td></td>
</tr>
<tr>
<td>• FOS</td>
<td></td>
</tr>
<tr>
<td>• Ensure during BH testing if needed,</td>
<td></td>
</tr>
<tr>
<td>• Provision of and assistance with placebo powder packaging</td>
<td></td>
</tr>
<tr>
<td>Feeding Study Design/Set-up</td>
<td>X</td>
</tr>
<tr>
<td>Counseling/Education:</td>
<td></td>
</tr>
<tr>
<td>Dietary</td>
<td></td>
</tr>
<tr>
<td>Body Composition</td>
<td></td>
</tr>
<tr>
<td>Nutrition Assessment:</td>
<td></td>
</tr>
<tr>
<td>Dietary Analysis (ESHA or NDSR)</td>
<td></td>
</tr>
<tr>
<td>Food Frequency Questionnaire</td>
<td></td>
</tr>
<tr>
<td>Total # of FFQ's requested:</td>
<td></td>
</tr>
<tr>
<td>Body Comp: Anthropometrics</td>
<td></td>
</tr>
<tr>
<td>Biompedance</td>
<td></td>
</tr>
<tr>
<td>Energy Expenditure (cart)</td>
<td></td>
</tr>
<tr>
<td>Data Calculations/Management:</td>
<td></td>
</tr>
<tr>
<td>Dietary</td>
<td></td>
</tr>
<tr>
<td>Body Composition</td>
<td></td>
</tr>
<tr>
<td>Energy Expenditure/Requirement</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
<tr>
<td>Assistance with Grant/Paper Writing</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
</tbody>
</table>

VIII. BIOSTATISTICAL REQUEST

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size/Power Calculation</td>
<td>N/A</td>
</tr>
<tr>
<td>Development of Statistical Methods</td>
<td>X</td>
</tr>
<tr>
<td>Analysis of Data</td>
<td>X</td>
</tr>
<tr>
<td>Preparation of a Statistical Report</td>
<td>X</td>
</tr>
<tr>
<td>Other</td>
<td>N/A</td>
</tr>
</tbody>
</table>
### IX. Core Laboratory Request

No core laboratory analyses requested. Co-investigator will be performing all analyses.

<table>
<thead>
<tr>
<th>Name Assay</th>
<th>Number of Subjects</th>
<th>Number of Tests per Subject</th>
<th>Total Number of Samples</th>
<th>Cost per Sample</th>
<th>Cost per Year</th>
<th>Cost during Lifetime of Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-Peptide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHEA Sulfate</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Estradiol</td>
<td></td>
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<tr>
<td>Free Insulin</td>
<td></td>
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</tr>
<tr>
<td>Glucagon</td>
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<tr>
<td>Glucose</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>GHG</td>
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<td></td>
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<tr>
<td>IGF-1</td>
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<tr>
<td>Insulin</td>
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<tr>
<td>Leptin</td>
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<tr>
<td>Osteocalcin</td>
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<tr>
<td>Plasma</td>
<td></td>
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<td></td>
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<tr>
<td>Catecholamines</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Cortisol</td>
<td></td>
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<td></td>
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<tr>
<td>Progestosterone</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Prolactin</td>
<td></td>
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<tr>
<td>Salivary Cortisol</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Sex Hormone, Rinfing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Specific specimen handling and labeling request(s), please:

1) Containers (and "popsicle stick") for stool collections (n = 48 containers and sticks), if possible. If not, investigator will identify source for these items.
2) Containers for urine collections (n = 48 containers).
3) Appropriate blood collection tubes n = 192 tubes (4 for each of 24 subjects X 2 visits).
4) Centrifuge plasma samples immediately after collection and place in vials provided by Human Nutrition for freezing. Freeze samples. Co-investigator will pick up promptly.
5) Labels for specimens (n = ___).

Is your grant budgeted to cover assay kit costs for these labs? Yes X, only partially. No.

NOTE: The values generated from the assays conducted by the GCRC Core Lab are to be used for research purposes only. These values are neither appropriate nor intended for clinical use.

Several analyses have been added to the protocol since the co-investigator originally approached Ross Labs for funding. The co-investigator is currently trying to secure additional funding for assay kits.
X. ANCILLARY ASSAY(S) REQUEST WORKSHEET (None, Thank you).

<table>
<thead>
<tr>
<th>Name of Test</th>
<th>Number Of Tests per Subject</th>
<th>Number of Subjects</th>
<th>Unit Cost (Admin use only)</th>
<th>Total Cost (Admin use only)</th>
</tr>
</thead>
</table>

XI. SPECIMEN HANDLING REQUESTS FOR OUTSIDE LABS

Please note that this is covered above. Co-investigator or microbiology graduate student will pick up all completed.

Contact information:

<table>
<thead>
<tr>
<th>Name</th>
<th>Title/Position</th>
<th>Phone</th>
<th>Email</th>
</tr>
</thead>
<tbody>
<tr>
<td>William B. Malarkey, M.D.</td>
<td>Program Director</td>
<td>614-293-8775</td>
<td><a href="mailto:malarkey-1@medctr.osu.edu">malarkey-1@medctr.osu.edu</a></td>
</tr>
<tr>
<td>David Phillips</td>
<td>Administrative Director</td>
<td>614-293-4406</td>
<td><a href="mailto:phillips-7@medctr.osu.edu">phillips-7@medctr.osu.edu</a></td>
</tr>
<tr>
<td>Diane Habash, Ph.D.</td>
<td>Bionutrition Manager</td>
<td>614-293-6669</td>
<td><a href="mailto:habash-1@medctr.osu.edu">habash-1@medctr.osu.edu</a></td>
</tr>
<tr>
<td>Susan Moseley</td>
<td>Core Lab Manager</td>
<td>614-293-4675</td>
<td><a href="mailto:moseley-1@medctr.osu.edu">moseley-1@medctr.osu.edu</a></td>
</tr>
<tr>
<td>&quot;Raj&quot; Nagaraja, Ph.D.</td>
<td>Biostatistician</td>
<td>614-293-4647</td>
<td>hnn@stat ohio-state.edu</td>
</tr>
<tr>
<td>Jacelynn Buck, Ph.D.</td>
<td>Director of Nursing</td>
<td>614-293-8749</td>
<td><a href="mailto:buck-1@medctr.osu.edu">buck-1@medctr.osu.edu</a></td>
</tr>
<tr>
<td>Robert Rice, Ph.D.</td>
<td>Informatics Manager</td>
<td>614-293-8614</td>
<td><a href="mailto:rice-1@osu.edu">rice-1@osu.edu</a></td>
</tr>
<tr>
<td>Carson Reider, Ph.D.</td>
<td>Research Subject Advocate</td>
<td>614-293-9274</td>
<td><a href="mailto:reider-1@medctr.osu.edu">reider-1@medctr.osu.edu</a></td>
</tr>
</tbody>
</table>

Submit your completed application to the GCRC Administrative Director, David Phillips, (phillips-7@medctr.osu.edu, N1105 Doan Hall, 410 West 10th Avenue).
ADDENDUM
(for GCRC use only)

Revisions to the GCRC Application per the GCRC Managers Meeting

Date of GCRC Managers Meeting review of Application: __________ / _______ / _______

NOTES:
APPENDIX C

LETTER TO PHYSICIANS
October 15, 2003

Dear Dr.

I am currently running a clinical trial for my doctoral dissertation entitled, “The Effect of Fructooligosaccharides (FOS) On The Absorption of Soy Isoflavones in Postmenopausal Women.” In this prospective randomized trial, I am investigating the effect of a fruit fiber (fructooligosaccharide- FOS) on the absorption of the primary active ingredient in soy protein, isoflavones, in the postmenopausal population. As you know, soy protein is associated with several health benefits (decreased LDL, improved blood pressure, maintenance of bone mineral density, and possibly relief from hot flashes). I have a packet of materials (my detailed research protocol with a literature review, my protocol written for OSU’s General Research Clinical Center- GCRC, and the OSU Institutional Review Board-approved Informed Consent and HIPAA forms) for your practice.

This study is being conducted at the GCRC and lasts 5 weeks. All the patients are given soy protein shakes during the treatment period, and randomized to either the FOS or a dextrose powder (placebo). The patients come in twice over the five weeks (week 2 and week 5) for an overnight stay where blood samples, fecal samples, urine samples and breath hydrogen samples are collected. I am currently recruiting patients from Dr. Kathy Lutter’s and Dr. Beth Boyle’s practices. Patients receive $100 for each of the two visits to the GCRC.

Patient eligibility requirements are as follow:

Twenty-four (24) healthy postmenopausal women will be recruited for this pilot study. The following criteria will be used for subject eligibility: 1) Subject has not taken any medication known to alter lipid, bone, or calcium metabolism including hormone replacement therapy over the past 6 months; 2) Subject has not had a menstrual period within the 12 months before the study is initiated but is not older than 70 years old; 3) Subject has no history of gastrointestinal or malabsorptive disorders (Crohn’s disease, ulcerative colitis, gluten enteropathy, irritable bowel syndrome, short bowel syndrome secondary to surgery, etc.); 4) Subject has not taken soy products for the three months and is not currently taking the type of soy and or isoflavone supplements ; 5) Subject has not taken fructooligosaccharide supplements for the past three months and is not currently taking fructooligosaccharide supplements for the past three months and is not currently taken antibiotics or probiotics for the past 3 months; 7) Subject is willing to be admitted to the GCRC two times, for approximately 20 hours each time over 5 consecutive weeks to obtain: a) urine samples for urine isoflavones; b) breath hydrogen samples; c) fecal samples for molecular characterization of the intestinal microflora; d) four (4) blood draws over 5 weeks for plasma selenium and soy isoflavones; and 8) Subject is willing to complete food records and gastrointestinal tolerance scales for two of the five weeks of the study. Subjects will be counseled to eliminate foods inherently high in soy or FOS (other than the soy and FOS treatment intervention supplements) from their diet.

Thank you so much in advance for your potential interest in this study. I look forward to possibly working with you on this exciting study in your highly-esteemed practice.

Warm regards,

Maureen E Geraghty, MS, RD,LD
Doctoral Candidate
Department of Human Nutrition
The Ohio State University Nutrition (OSUN) PhD program
APPENDIX D

PATIENT MATERIAL
CONSENT TO INVESTIGATIONAL TREATMENT OR PROCEDURE

I, _______________________, hereby authorize or direct Dr. Anne Smith PhD, RD, LD and associates or assistants of his/her choosing, to perform the following treatment or procedure (describe in general terms).

The purpose of this study is to evaluate whether the fruit fibers called fructooligosaccharides (FOS) help the active ingredients of soy protein (called isoflavones) to be absorbed by the body. This will be determined by:

1) measuring components of soy protein in the urine; 2) measuring blood isoflavones; 3) applying special microbiology molecular techniques to stool samples to determine if there is an increase, decrease, or no change in the good bacteria in the intestine after the FOS is consumed.

Fructooligosaccharide (FOS) is a naturally occurring polymer of fructose (fruit sugar) attached to a glucose molecule. It is considered a soluble fiber (it dissolves in water). FOS is found in many fruits, vegetables, and grains. FOS powder can be made by an enzymatic action (a simple chemical reaction) with sucrose (table sugar). I would have to eat six bananas, 4 onions, or 95 cloves of garlic daily to get the equivalent of ¼ tsp (1 gram) of FOS powder.

At the initial (screening) visit, a registered dietitian (the co-investigator) will carefully review my medical history and medications to determine if I qualify for the study. If I qualify for the study, the dietitian will schedule my Baseline Visit and I will remain on the study for five weeks. This study has two periods:

1) Baseline Period (2 weeks)
2) Treatment Period (3 weeks)

I will be admitted to the Ohio State University (OSU) General Clinical Research Center (GCRC) during the last week of the two periods for approximately 20 hours. (from 6:00 p.m. on Wednesday evening until 2:00 pm on Thursday)

Following is a figure that shows where the GCRC admissions take place during this 5 week study:
Admissions depicted by ▼ below:

Baseline Period ▼ → Treatment Period ▼
(Weeks 1-2) (Weeks 3-5)

I will be admitted on 6:00 PM of the Wednesday evening during the last week of each period for a total of two GCRC admissions. I will be asked to fast from 6:00 PM on Wednesday (upon admission) until 6 AM on Thursday morning. I will be asked to bring a stool sample in from Wednesday if possible. (If not, I will try to provide a stool sample while in the GCRC). Starting at 6 AM on Thursday, I will start collecting a 24-hour urine
sample and I will be asked to provide up to a total of 6 breath samples and up to a total of 4 blood samples until 2:00 PM on Thursday. Approximately 24 postmenopausal women will participate in this study. Should I have any questions regarding my participation associated with the study activities on the OSU General Clinical Research Center (GCRC), I may contact the Research Subject Advocate for the OSU GCRC at (614) 293-9274.

upon ___________________________.

(myself or name of subject)

The experimental (research) portion of the treatment or procedure is:

This study will be a prospective randomized parallel trial. Prospective means that I will be followed in the study for 5 weeks in the near future, and randomized means that the order in which A or B are given to me will be determined by chance, such as a flip of a coin. Parallel means that some subjects will be on Treatment A while others will be on Treatment B during the course of the study. I will be randomly assigned to only one treatment- A or B, during the Treatment Period. (If on Treatment A, I will prepare and drink a soy shake every morning and take a sugar powder (placebo) 3 times a day; if on Treatment B, I will prepare and drink and soy shake every morning, AND take FOS powder 3 times a day.

During the Baseline Period 1 visit, the dietitian will counsel me to avoid foods or dietary supplements high in soy or FOS throughout the study (other than the soy or FOS treatments taken during the Treatment Period. During this Baseline Period, I eat what I normally eat without going on any treatment. It is during the Treatment Period that I am randomly assigned to take one of the treatments: Treatment A or B.

Treatment A, the soy shake, is made from Health Source® Soy Protein Powder (Ross Products Division, Abbott Laboratories, Columbus, OH) and a placebo (sugar) powder (Sigma Chemical, St. Louis, MO). Two scoops of the powder (26 teaspoons) are added to 8 ounces of skim milk (or 2% milk) to make the shake. The flavor of the shake is chocolate, and other items such as strawberries, chocolate syrup, etc. can be added to enhance the flavor. Treatment B is the soy shake AND the FOS powder (GOS powder from GTC Nutrition, LLC, Golden, CO). If I am on Treatment B, I will be started on ¼ teaspoon of FOS and it will be gradually increased to 2 ½ tsp. a day. The gradual dosing will help my intestines adjust to the FOS, which is a fiber. I can add the FOS to a noncarbonated drink as a way to take it. The FOS itself has no taste. The soy shakes are taken in the mornings of the Treatment Period. Also during the Treatment Period I will consume my FOS or placebo powder in three divided doses throughout the day.

During the four days before I am admitted to the GCRC each period, (Saturday, Sunday, Monday, and Tuesday) I will keep 4-day food records and 4 day gastrointestinal tolerance records. On that Wednesday of admission, I will also try to collect a stool sample in a container provided by the co-investigator at the Baseline 1 visit. On the Wednesday evenings before my 6 PM GCRC admission, I will consume a dinner consisting of one 8-fluid ounce (240 mL) can of Ensure Plus with an individualized number of Ensure® Nutrition and Energy Bars to provide 1/3 of my daily estimated energy or caloric requirement. This meal is known to produce very little hydrogen during digestion and so it
will make the breath hydrogen tests more accurate the next day. The Ensure ® liquid and bars will be provided to me. Following this meal, I will begin a period of fasting for 12 hours during which I will be allowed to consume water as desired, but no smoking will be permitted. Upon waking the morning of each “Test Thursday” (the Thursdays of the two GCRC admissions), I will be asked to drink 4-6 ounces of water.

The following procedures will be performed during the two GCRC admissions:
Measurement of weight, height, review of my 4-day diet and gastrointestinal tolerance records, and measurement of vital signs- temperature, blood pressure, and heart rate (TPRs) after at least 30 minutes of rest. Blood samples will also be taken twice during each admission for a total of 4 blood draws. During the week prior to each of my two admissions, I will complete four days of dietary intake records (two weekdays and two weekend days) and I will complete Gastrointestinal Tolerance Scales on the same four days.

The other measurements will be taken at the specified visits as follows during my visits to the GCRC: (Meals are also listed). I am admitted Wednesday evening at 6:00 PM and will fast until Thursday morning. Following is a table as to what to expect on both the Baseline and Treatment Visits.

**Baseline Period**-Sample Collections and Meals in GCRC (Week 2)

<table>
<thead>
<tr>
<th>Test Thursday</th>
<th>I will:</th>
</tr>
</thead>
<tbody>
<tr>
<td>6:00am</td>
<td>Provide one breath hydrogen sample.</td>
</tr>
<tr>
<td>6:00am</td>
<td>Start a 24-h urine collection. Try to provide stool sample sometime today.</td>
</tr>
<tr>
<td>8:00am</td>
<td>Have blood drawn (1 4/5 tsp).*</td>
</tr>
<tr>
<td>8:00am</td>
<td>Be served a regular breakfast (Soy and FOS-free).</td>
</tr>
<tr>
<td>12 noon</td>
<td>Be served a regular lunch (Soy and FOS-free).</td>
</tr>
<tr>
<td>2:00 pm</td>
<td>Have blood drawn(1 4/5 tsp).*</td>
</tr>
<tr>
<td>2:15pm</td>
<td>Be discharged home. Arrangements will be made for the Co-investigator to pick up 24 –h urine sample (and stool sample if I am not able to provide it in the GCRC).</td>
</tr>
</tbody>
</table>

**Treatment Period**-Sample Collections and Meals in GCRC (Week 5)

<table>
<thead>
<tr>
<th>Test Thursday</th>
<th>I will:</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:45am</td>
<td>Provide breath hydrogen sample.</td>
</tr>
<tr>
<td>6:00 am</td>
<td>Provide breath hydrogen sample.</td>
</tr>
<tr>
<td>6:00 am</td>
<td>Start 24-h urine collection. Try to provide stool sample sometime today.</td>
</tr>
<tr>
<td>6:01 am</td>
<td>Drink Soy shake</td>
</tr>
<tr>
<td>6:30 am</td>
<td>Take Treatment A or B powder. A= placebo (sugar); B = FOS.</td>
</tr>
<tr>
<td>8:00 am</td>
<td>Provide breath hydrogen sample.</td>
</tr>
<tr>
<td>Time</td>
<td>Activity</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>8:00 am</td>
<td>Have blood drawn (1 4/5 tsp).*</td>
</tr>
<tr>
<td>10:00 am</td>
<td>Provide Breath Hydrogen sample.</td>
</tr>
<tr>
<td>11:00 am</td>
<td>Drink 1 can Ensure Plus if desired.</td>
</tr>
<tr>
<td>12 noon</td>
<td>Provide breath hydrogen sample.</td>
</tr>
<tr>
<td>2:00 pm</td>
<td>Provide breath hydrogen sample.</td>
</tr>
<tr>
<td>2:00 pm</td>
<td>Have blood drawn (1 4/5 tsp).</td>
</tr>
<tr>
<td>2:15 pm</td>
<td>Be served lunch.</td>
</tr>
<tr>
<td>2:30 pm</td>
<td>Be discharged to home. Arrangements will be made with the Co-investigator to pick up the 24-h urine and (stool sample if I am not able to provide one in the GCRC.</td>
</tr>
</tbody>
</table>

* 8 am and 2 pm for each admission 9cc of blood is drawn, which = 1 4/5 tsp.
As a reference, 10 cc of blood is equal to 2 teaspoons.

This is done as part of an investigation entitled: “The Effect of a Fruit Fiber on the Intestinal Absorption of Isoflavones from Soy Protein in Postmenopausal Women.”

1. **Purpose of the procedure or treatment:**
The main purpose is to determine whether the addition of fruit fiber (FOS) can enhance the absorption of isoflavones (the active ingredient in soy) in the body. Secondary purposes include: 1) examining the changes that may occur in the good bacteria (enzyme changes and quality and quantity changes in the bacteria (through a stool sample) when on one of the two treatments (soy with placebo or soy with FOS); 2) to determine if there is a relationship between breath hydrogen values and bacteria in the stool; 3) to evaluate whether the two treatments change plasma isoflavone or selenium levels; 4) to evaluate daily protein intake of the subjects through diet records while on the treatment (Treatment Period) and while not on the treatment (Baseline Period); and to measure gastrointestinal tolerance (presence or absence of bloating, gas, etc. of the two treatments using a written scale. The term *plasma isoflavone* refers to the active ingredient of soy (isoflavones) circulating in your blood (the plasma portion of your blood). *Selenium* is a trace mineral and essential nutrient that acts within proteins as an antioxidant.

2. **Possible appropriate alternative procedure or treatment (not to participate in the study is always an option):**
There is no alternative procedure other than non-participation in the study.

3. **Discomforts and risks reasonably to be expected:**
The risks of this protocol are minor and may be associated with blood sampling, fasting, or with possible gastrointestinal side effects of the treatments (soy + placebo powder or soy and the fruit fiber-FOS). An attending physician will be available to me in the OSU General Clinical Research Center (GCRC) to assume medical record responsibilities, and
to address other medical needs, should they arise. A registered nurse will perform the blood draws using sterile technique with appropriate medical supplies and will exercise universal precautions pertaining to the procedure. Possible side effects of venous blood draws include pain, swelling, redness, bruising, and rarely, an infection or clotting at the indwelling catheter site. An indwelling catheter is a small tube inserted under the skin that allows for multiple blood draws without “sticking” the subject with a needle each time. Fasting can cause dizziness, headaches, stomach discomfort, and fainting. Breath sampling is a noninvasive procedure and carries no known risk, although there may be some discomfort such as dizziness, or headaches associated with the fasting that is involved. I understand that during the breath hydrogen sampling during the Treatment Period, I can take 1-2 cans of Ensure Plus (a commercial liquid nutritional product from Ross Products Division) after 10 AM to ease the hunger until 2:00 PM when the testing is over. I will then be served a regular lunch. All blood draws by nursing and fasting by subjects will take place only while I am in the GCRC. Possible side effects from the soy shake or the FOS (fruit fiber) include nausea, cramping, diarrhea, gas, or bloating. These gastrointestinal side effects may be more likely with the FOS powder; they are not very common with soy in terms of frequency or severity. If I do experience some discomfort with the FOS, the 2 ¼ teaspoon daily dose can be decreased to help me tolerate it better. Both the Nutraflora® FOS (fructooligosaccharide) powder and the Health Source® Soy Protein Shake Powder are commercially available. The Nutraflora® FOS is the only FOS product in the United States to have “Generally Regarded AS Safe” –GRAS recognition (GRAS #GRN000044).

Potential inconveniences to me as a subject in this study may include the following: 1) time to participate in the study for which I must be admitted to the GCRC two times for 20 hours each; 2) the period of overnight fasting (the Weds. night of both GCRC admissions) prior to each GCRC visit, and the period of fasting after the Thursday morning soy shake or soy shake + FOS during the Treatment Period GCRC visit; 3) gastrointestinal symptoms which may develop from consuming the treatment products; 4) discomfort associated with the blood sampling procedure; 5) time associated with the completion of diet records and gastrointestinal tolerance records, as well as the time and effort of trying to collect a stool sample the Wednesday before the evening GCRC admissions at the end of both Periods. The Co-investigator, a registered and licensed dietitian, (Maureen Geraghty, MS,RD,LD, can be contacted at 395-1430 in the event discomfort is experienced with the FOS. She will instruct me as to how to reduce my dose to a more tolerable level.

Steps that will be taken to protect my right to confidentiality as a subject include: 1) Data from the study will only be reported in a confidential fashion using subject identification numbers and initials, not my full name. All screening information and other subject information will be kept in a locked cabinet in the co-investigator’s office at all times when not in use. Study records that identify me will be kept confidential and, to the extent permitted by applicable laws and regulations, will not be made publicly available. Since a portion of this research project will be conducted at the OSU GCRC, the Program/Medical Director, Research Subject Advocate, and/or other members(s) of the nursing staff may
review my medical record. A copy of this written informed consent has been given to me for my own records and reference.

In the event I am a student or an employee at The Ohio State University, my grades or relationship with the University will not be affected if I choose to withdraw from or not participate in the study for any reason.

There will be no charge to me to participate in this study. The treatments (Soy powder and FOS) and the pre-admission meal that precedes fasting (Ensure® nutritional liquid and bars) will be provided free of charge. All studies will be conducted at no charge to me.

4. Possible benefits for subjects/society:
There is no intended clinical benefit to me for participating in the study, although long term clinical benefits (greater than four consecutive weeks) have been documented for both soy and FOS. This knowledge and familiarization I will have with these functional foods may lead to a longer term consumption of one or both. Long term consumption of soy is associated with such benefits as reduced serum cholesterol, prevention of bone loss, and protection from some cancers. Some people have seen an improvement in their serum cholesterol levels after one month consumption at the study dose. The FDA has approved the statement on soy products that, “Soy Protein May Reduce Your Risk of Heart Disease” based on a dose of 25 grams of soy protein per day. In this study, I will be consuming 26 grams of soy protein per day. Along with other nutrients, this soy product also provides 600 milligrams of calcium per serving.

Fructooligosaccharides can have a positive impact on overall health (via increasing the balance of the friendly bacteria in the bowel) by improving bowel regularity, by enhancing calcium and magnesium absorption by the body, and by helping to lower cholesterol.

The primary benefit derived from this study for society will be the knowledge as to whether FOS can enhance absorption of soy in postmenopausal women by increasing the friendly bacteria in the bowel. If this is the case, a larger trial can be designed, and ultimately medical nutrition recommendations for the addition of FOS to one’s daily soy regimen may be published in the scientific literature.

5. Anticipated duration of subject's participation (including number of visits):
There are two study periods: 1) Baseline Period (2 weeks); and the Treatment Period (3 weeks). I will complete a total of two visits to the GCRC (approximately 20 hours per visit) at the end of both periods. Before the GCRC visits, I will have a screening in my gynecologist’s office which will last approximately 45 minutes to one hour. The exit visit will be considered the last 15 minutes of the second GCRC visit. I will also spend about 15-20 minutes per day for 8 days of diet records and gastrointestinal tolerance records. My total duration of participation in this study will last 5 weeks (35 days) unless a GCRC visit has to be postponed a week to accommodate my schedule.
I understand that the study investigator or doctor may remove me from the study at any time without my consent for any of the following reasons: 1) They decide discontinuation of the study is in my best medical interest; 2) I fail to follow study directions; 3) I fail to consume the entire allotted treatment during the Treatment Period, 4) I no longer meet study qualification requirements; 5) study is cancelled; or 6) the targeted number of subjects has been started in the study.

After completing the study (both GCRC visits), and turning in all requested diet and gastrointestinal tolerance records, I will be paid $200. If I withdraw before completing the study, I will receive $100 per completed GCRC test visit. (This includes turning in all the diet and gastrointestinal records per visit.) I will not be paid for the screening visit.

I hereby acknowledge that _______________________________ has provided information about the procedure described above, about my rights as a subject, and he/she answered all questions to my satisfaction. I understand that I may contact him/her at Phone No. 292-0715 should I have additional questions. He/She has explained the risks described above and I understand them; he/she has also offered to explain all possible risks or complications.

I understand that, where appropriate, the U.S. Food and Drug Administration may inspect records pertaining to this study. I understand further that records obtained during my participation in this study that may contain my name or other personal identifiers may be made available to the sponsor of this study. Beyond this, I understand that my participation will remain confidential.

I understand that I am free to withdraw my consent and participation in this project at any time after notifying the project director without prejudicing future care. No guarantee has been given to me concerning this treatment or procedure.

I understand in signing this form that, beyond giving consent, I am not waiving any legal rights that I might otherwise have, and I am not releasing the investigator, the sponsor, the institution, or its agents from any legal liability for damages that they might otherwise have.

In the event of injury resulting from participation in this study, I also understand that immediate medical treatment is available at University Hospitals of The Ohio State University and that the costs of such treatment will be at my expense; financial compensation beyond that required by law is not available. Questions about this should be directed to the Office of Responsible Research Practices at (614) 688-4792.

I have read and fully understand the consent form. I sign it freely and voluntarily. A copy has been given to me.
I certify that I have personally completed all blanks in this form and explained them to the subject or his/her representative before requesting the subject or his/her representative to sign it.

Date: _______ Signed _____________________________

(Signature of Project Director or his/her Authorized Representative)

HS-028A (Rev. 7/93)
Attention Healthy Postmenopausal Women:

Do you:

- Have concerns about your health?
- An interest in soy but are not currently taking soy?
  
  A Women’s Health Study is being conducted in healthy postmenopausal women to test how soy is absorbed in the body when given with and without a special fruit fiber.

Qualified Participants will receive free:

- Confidential study-related medical exam
- Blood tests
- Soy powder
- Fruit fiber powder
- Dietary analyses by a registered dietitian

Some qualification requirements for participation include that you:

- Have been postmenopausal for at least one year
- Are between 50 - 70 years old (inclusive)
- Have never been on/or have discontinued hormone replacement therapy (HRT) for the past 6 months.
- Have no chronic diseases
- Are not currently taking medications that would alter absorption or metabolism of nutrients

Financial Compensation is available for your time and for parking expenses.

If you are interested and want to be further evaluated for qualification as a research subject on this diet study, please call:

**Anne Smith, PhD, RD, LD**

(614)292-0715

Dept of Human Nutrition, College of Human Ecology

In conjunction with

General Clinical Research Center

The Ohio State University

450 W 10th Ave.

Columbus, Ohio 43210
Title of the Study:
“The Effect of a Fruit Fiber on the Intestinal Absorption of Isoflavones from Soy Protein in Postmenopausal Women.”

OSU Protocol Number: 2003H0002

Principal Investigator: Anne Smith, PhD RD, LD
Co-investigator: Maureen Geraghty, MS, RD,LD

Subject Name__________________________________________________________

Before researchers use or share any health information about you as part of this study, The Ohio State University is required to obtain your authorization. This helps explain to you how this information will be used or shared with others involved in the study.

• The Ohio State University and its hospitals, clinics, health-care providers and researchers are required to protect the privacy of your health information.

• You should have received a Notice of Privacy Practices when you received health care services here. If not, let us know and a copy will be given to you. Please carefully review this information. Ask if you have any questions or do not understand any parts of this notice.

• If you agree to take part in this study your health information will be used and shared with others involved in this study. Also, any new health information about you that comes from tests or other parts of this study will be shared with those involved in this study.

• Health information about you that will be used or shared with others involved in this study may include your research record and any health care records at the Ohio State University. For example, this may include your medical records, x-ray or laboratory results. Psychotherapy notes in your health records (if any) will not, however, be shared or used. Use of these notes requires a separate, signed authorization.

Please read the information carefully before signing this form. Please ask if you have any questions about this authorization, the University’s Notice of Privacy Practices or the study before signing this form.

Initials/Date: __________________

Page 1 of 3
Those Who May Use, Share And Receive Your Information As Part Of This Study

- Researchers and staff at The Ohio State University will use, share and receive your personal health information for this research study. Other Ohio State University staff not involved in the study but who may become involved in your care for study-related treatment will have access to your information.
- Those who oversee the study will have access to your information, including:
  - Members and staff of the Ohio State University’s Institutional Review Boards, including the Western Institutional Review Board
  - The Office for Responsible Research Practices
  - University data safety monitoring committees
  - The Ohio State University Research Foundation
- Your health information may also be shared with federal and state agencies that have oversight of the study or to whom access is required under the law. These may include:
  - The Food and Drug Administration
  - The Office for Human Research Protections
  - The National Institutes of Health
  - The Ohio Department of Human Services

These researchers, companies and/or organization(s) outside of The Ohio State University may also use, share and receive your health information in connection with this study:

- The companies who provided product support: Ross Products Division (Division of Abbott Laboratories - Columbus, Ohio), who provided the Health Source® soy shake for the study, and GTC Nutrition LLC, (Golden Colorado), who provided the Nutraflora® fruit fiber for the study.

- Others: Dr. Kathleen Lutter, MD, and associates with Northwest Obstetrics and Gynecology Associates, 3217 Ridge Mill Dr., Hilliard, OH 43026; Dr. Beth Boyles, MD, and associates with Women Physicians in OB/GYN, 3545 Olentangy River Road, Suite 525, Columbus, OH 43214; Dr. Donna T. Diaz, MD and associates with Paraskos, Teteris, & Diaz, MD, INC. 3600 Olentangy River Road, Building 490, Columbus, OH 43214.

The information that is shared with those listed above may no longer be protected by federal privacy rules.

Initials/Date______________
Authorization Period

This authorization will not expire unless you change your mind and revoke it in writing. There is no set date at which your information will be destroyed or no longer used. This is because the information used and created during the study may be analyzed for many years, and it is not possible to know when this will be complete.

Signing the Authorization

- You have the right to refuse to sign this authorization. Your health care outside of the study, payment for your health care, and your health care benefits will not be affected if you choose not to sign this form.
- You will not be able to take part in this study and will not receive any study treatments if you do not sign this form.
- If you sign this authorization, you may change your mind at any time. Researchers may continue to use information collected up until the time that you formally changed your mind. If you change your mind, your authorization must be revoked in writing. To revoke your authorization, please write to:
  
  Anne M. Smith, PhD, RD, LD, The Ohio State University, Dept. of Human Nutrition, 325 Campbell Hall, 1787 Neil Ave. Columbus, OH 43210-1295. Phone: 292-0715

- Signing this authorization also means that you will not be able to see or copy your study-related information until the study is completed. This includes any portion of your medical records that describes study treatment.

Contacts for Questions

- If you have any questions relating to your privacy rights, please contact Margaret L. Johnson, J.D., HIPAA Privacy Manager, The Ohio State University, 140 Doan Hall, 410 W. Tenth Ave. Columbus, Ohio 43210.
- If you have any questions relating to the research, please contact Anne M. Smith, PhD, RD, LD. The Ohio State University, Dept of Human Nutrition, 325 Campbell Hall, 1787 Neil Ave., Columbus, Ohio, 43210-1295. Phone: 292-0715.

Signature

I have read (or someone has read to me) this form and have been able to ask questions. All of my questions about this form have been answered to my satisfaction. By signing below, I permit Dr. Anne Smith, PhD, RD, LD and the others listed on this form to use and share my personal health information for this study. I will be given a copy of this signed form.

Signature________________________________________________________
(Subject or Legally Authorized Representative)

Name _____________________________________________________________
(Print name above)
(If legal representative, also print relationship to subject.)
Date______________ Time _________ AM / PM

Page 3 of 3
Clinical Trial Participant Packet for
The Soy and Fruit Fiber Study (NUTR23)

Participant’s name:  *Name*

Dietitian and Clinical Study Co-investigator:
Maureen Geraghty, MS,RD,LD

Cell:  395-1430
Answering machine:  985-5110
Emails :  
geraghtv.6@osu.edu
megeraghty@aol.com
Thank you very much for agreeing to participate in this 5 week nutrition study. During the study, there are two occasions on which you will be admitted to the GCRC for “sample collections” for the study. (This means urine samples, blood samples, stool samples and breath samples). There are two periods in this study. A graphic depiction of the periods may help make the study easier to understand:

<table>
<thead>
<tr>
<th>Period 1</th>
<th>Period 2</th>
<th>Off study</th>
<th>Off study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (Wks 1-2)</td>
<td>Treatment Period (Wks 3-5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

You will be admitted to the GCRC on the Wednesday evening of the last week of each period, and you will be discharge approximately 20 hours later on Thursday afternoon. For example, for the Baseline Period, you be admitted to the GCRC during the second week of the study. During the Treatment Period, you will come in during the 5th week of the study.

Maureen, your dietitian and study co-coordinator, will give you a calendar with all the important dates on it.
The next few pages of this packet tells you what happens during each period of the study, for when you are at home, and then when you are admitted to the GCRC.

**Baseline Period:**

**Week 1:**

Dates: __________________________

**Monday**

Simply follow baseline diet (usual diet with no high soy or high fruit fiber foods or supplements)

**Tuesday**

Same as Monday.

**Wednesday**

Same as Monday.

**Thursday**

Same as Monday.

**Friday**

Same as Monday

**Saturday**

Begin 4-day Food Records and Gastrointestinal Tolerance Records (in this packet)

**Week 2:**

Dates: __________________________

**Sunday**

Continue with 4-day Food Records and Gastrointestinal Tolerance Records.

**Monday**

Continue with 4-day Food Records and Gastrointestinal Tolerance Records.

**Tuesday**

Complete last day of 4-day Food Records and Gastrointestinal Tolerance Records.

**Wednesday**

Start trying to collect a stool sample. (Only about 2 Tablespoons needed- stool collection kit will be provided by dietitian.) Usual breakfast and lunch for the study period. An early dinner (test meal) is to be eaten before coming to the hospital. This is to prepare for breath hydrogen testing.

**Test meal = 1 can Ensure Plus and _____ Ensure Bars.**

(Maureen will calculate how many you need).

You will start fasting after your early dinner. Water is allowed.

Please remember:

- 6:00 pm on Wednesday evening, is your GCRC admission time.

**For your GCRC visit, please bring:**

1) Diet records
2) Gastrointestinal Tolerance Records
3) Stool sample if available.
Baseline GCRC visit:

**Wednesday**

Date: _________________________

6:00 pm  Admission to GCRC. (Parking Pass and Map will be provided). Maureen will meet you in the lobby of the Ohio State University Medical Center.

- Please maintain fasting until Thursday morning.
- Height and weight will be recorded by nursing.

**Thursday:**

Date: _________________________

6:00 am  You will be asked to provide one breath hydrogen sample. (This is a non-treatment period, so only one breath sample is needed.)

24-hour urine collection is started.

You will be reminded to provide a stool sample is one not already obtained from Wednesday.

8:00 am  Blood will be drawn

You will be served a regular breakfast free of SOY and FOS.

NOON You will be served a regular lunch free of SOY and FOS.

2:00 pm  Blood will be drawn

2:15 pm  A snack will be served if desired.

2:30 pm  Discharged to home ☺

**Note:** On Thursday, your Dietitian/Study Co-investigator will visit you. She will review your food and gastrointestinal records with you and will provide diet counseling and nutritional products for Treatment period (which will start Sunday). You will continue your urine collection at home until 6 a.m. Friday morning. The Dietitian/study Co-investigator will pick urine collection up at your home. If stool collection is not yet provided, it will be picked up at the same time as the urine sample.

**Saturday:**  Continue Baseline diet.

**Sunday:**  Start Treatment Period
Treatment Period:

Week 3 Dates: _________________________________

Sunday-Saturday
This is considered a nutrition treatment period. Please follow the Treatment Period instructions given by dietitian last Thursday. You will have a soy milkshake for breakfast and the either the FOS powder or the placebo powder. (These powders can be added to noncarbonated drinks per your preference.)

Week 4 Dates: _________________________________

Sunday-Friday
Same as Week 3.

Saturday Date: _________________________________

Continue Treatment Period Diet as above.
Please begin 4-day Food Records and Gastrointestinal Tolerance Records.

Week 5 Dates: _________________________________

Sunday-Tuesday
Continue Treatment Period Diet
Continue Food Records and Gastrointestinal Tolerance Records.

Wednesday
Start collecting trying to collect stool sample.
Follow Treatment Period diet for Breakfast and Lunch.
An early dinner (test meal) is to be eaten before coming to the hospital.
Water is allowed.

Test meal = 1 can Ensure Plus and _____ Ensure Bars.

Please remember:

6:00 pm on Wednesday evening is your GCRC admission time.
For your GCRC visit, please bring:

1) Diet records
2) Gastrointestinal Intolerance Record
3) Stool sample if available
Treatment Period GCRC Visit

Wednesday (Continued)  Date: _______________________________

6:00 p.m.  Admission to GCRC.  (Parking Pass and Map will be provided)
  • Please maintain fasting. Water is still allowed.
  • Weight will be recorded by nursing.

Thursday  Date: _______________________________

You will be asked to provide 6 breath samples, please, starting at 5:45 a.m.

5:45 am  Baseline breath hydrogen sample collected

6:00 am  Baseline breath hydrogen sample collected

6:00 am  Start 24-h urine collection

6:01 am  Soy milkshake

6:30 am  Treatment A or B powder, A= placebo, B = FOS (Blinded.)

8:00 am  Breath hydrogen sample collected

8:00 am  Blood drawn

10:00 am  Breath hydrogen sample collected

11:00 am  I can of Ensure Plus if desired

NOON  Breath hydrogen sample

2:00 pm  Breath hydrogen sample

2:00 pm  Blood drawn
  Regular lunch served.

2:30 pm  Discharged to home 😊

Please Note:  You will continue your urine collection at home until 6 a.m. Friday morning. The Dietitian/study Co-investigator will pick urine collection up at your home. If stool collection is not yet provided, it will be picked up at the same time as the urine sample.

Congratulations! You have successfully completed this clinical study! Thank you for all your time and for contributing to the science of medical nutrition!
APPENDIX E

GUIDELINES FOR FOLLOWING A “SOY-FREE” DIET
Guidelines for following a “Soy-free” diet

Soy Products

♦ Soy milk
♦ Soy Protein Isolate (found in many Soy Powders)
♦ Soy Bars
  (Brand names such as Genisoy,™ Health Source,™ are often found on bars and powders)
♦ Meat alternatives, such as TSP- (Textured Soy Protein) or TVP- (Textured Vegetable Protein). These are found in veggie burgers, veggie chili, veggie sausages, etc. Worthington Foods is a local distributor of vegetarian meat alternative products.
♦ Soy nut butter
♦ Soy nuts
♦ Whole Soybeans, roasted or broiled
♦ Miso
♦ Tofu
♦ Tempeh
♦ Soy flour
♦ Isoflavone supplements
♦ Soy bean oil (hydrogenated or partially hydrogenated)*

* This last item can be difficult to omit from one’s diet since so many items contain soy bean oil.
APPENDIX F

DIETITIAN/CO-INVESTIGATOR'S CHECKLIST FOR SCREENING/INITIATION VISIT
Dietitian/Co-investigator’s checklist
For
Screening/Initiation Visit

☐ Screening Form
☐ HIPAA Form
☐ Informed Consent
☐ Clinical Trial Participant Packet:
  • Graphic depiction of visits/Written description of each visit with specific dates, entitled, “All about the Four Study Periods and Visits to the GCRC”
  • Diet Guidelines (For Soy-free, FOS-free and to explain protein/calorie contribution of supplements.
  • Food Records w/dates
  • Gastrointestinal Tolerance Records w/dates
  • Map/Parking Passes

☐ Clinical Trial Edible items: (Interventions and pre-admission
  Ensure test meal items)
  • Soy powder w/shaker
  • Ensure Plus (for dinner before the Weds admissions)
  • Ensure Bars (for dinner before the Weds admissions)

☐ Clinical Trial/Sample Collection Supplies
  • Urine/Stool collection “hats”
  • Gloves, Stool transfer tube (15 mL Falcon tube)
  • Sterile thin popsicle sticks

☐ Reassure subject that RD will give a reminder phone call on four designated dates (the Friday preceding the Saturday when she will keep the 4-day Food Records and Gastrointestinal Tolerance Records.) She will also call subject the Tuesday preceding the Wednesday GCRC admission to answer any questions.

Subject Number: ___________________
Date: ___________________

Notes:
APPENDIX G

K. WALS H'S URINARY ISOFLAVONOID EXTRACTION
PROTOCOL FOR HPLC
K. Walsh's* Urinary Isoflavonoid Extraction Protocol for HPLC  
(Six samples/run)

DAY 1:
1. Thaw urine at room temperature.
2. Transfer 8 mL aliquot to 50 mL centrifuge tube.
3. Centrifuge at 8500 RPM (JA-17 rotor) for 10 min @ 4°C.

Vacuum Manifold
4. Prep Sep pack C18 columns on vacuum manifold (designed by K Walsh in Dr. Mark Failla’s lab)
   a) wash with 6 mL MeOH
   b) wash with 6 mL acetate buffer (pH 4.0)
5. Centrifuged urine: filter 5 mL through 0.45 μm nylon filter placed on top of C-18 column (keep vacuum
   manifold lever closed).
6. Add 16.5 μL 2',4'-dihydroxy-2-phenylacetophenone (DHPAP)
7. Add 1.5 mL acetate buffer (pH 4.0)
8. Filter samples through column, discard waste. (OPEN manifold lever and turn on water attached to
   vacuum).
9. Wash with 4mL acetate buffer.
10. Wash with 1 mL diH2O
11. Wash with 1 mL MeOH, discard waste.
12. Elute compounds into 11 mL glass vials w/ 7 mL MeOH
13. Rinse columns with 8 mL acetone (spray bottle) after use.
14. Dry samples under N2 at 37°C.
15. Dissolve residue in 1 mL β-glucuronidase (460 μg/ 1mL sodium acetate buffer)
16. Incubate overnight at 37°C in rocking incubator @ 40 rpm.

Day 2:
17. Add 3.5 mL diethyl ether to each sample (under hood)
18. Shake by hand 20 sec and extract top phase with transfer pipette
19. Repeat 2 more time with 3.5 mL diethyl ether.
20. Dry under N2. Freeze at -80 until analyzed.

For HPLC Analysis: Resolubilize in 600 μL 80% MeOH; Filter and inject.
* Kelly developed method with direction from the Dept. of Food Science and Technology.
APPENDIX H

DGGE PROCEDURE-SYNOPSIS
DGGE Procedure-synopsis

Written by Maureen Geraghty
With assistance from John Sylvester and Sanjay Karnati.

Photos From left to right:
A. Core (yellow casing); B. Casting Stand, C. Perpendicular stand not used in this lab; D. Gradient Former; E. Electrophoresis Temperature Control Module & Tank.
(Photo used for educational purposes from www.biorad.com)

DGGE Procedure:

Prep: Gel plates/combs, spacers
  • Clean plate glass and combs/spacers w/ 70% EtOH
  • Large glass in back, short glass in front

Prep: GEL:
  • High and Low – 50 mL Falcon tubes- one each for one gel, 2 ea.
• Place in ice bucket. Usually have 2-50 mL tubes each for 2 gels.

• Will need specific concentrations of 7.5% acrylamide – 100% Formamide (40% formamide and 7M urea) and 7.5% acrylamide-0% Formamide (no formamide or urea). See recipes last page if no more stock solution. Stock solutions in brown bottles in 4°C fridge/or with covered with foil.

• If use stock solution, place in 37°C water bath for 30 min. before using.

• Example of calculation:

  Gel: 40%-60%:

  Need 16 mLs. total: \[
  \begin{align*}
  &16 &100 \\
  &X &40 ; &X &6.4 \text{ mLs} , &X &60 ; &X &9.6 \text{ mLs} .
  \end{align*}
  \]

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Low (40%)</th>
<th>High (60%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>6.4 mLs</td>
<td>9.6 mLs</td>
</tr>
<tr>
<td>0%</td>
<td>9.6 mLs</td>
<td>6.4 mLs</td>
</tr>
<tr>
<td><strong>Total mLs.</strong></td>
<td>16.0 mLs</td>
<td>16.0 mLs</td>
</tr>
</tbody>
</table>

Assembly of DGGE equipment:

• Spacers on end of glass. Spacer w/ slit goes on right. (between small and tall glass.) Spacer- “Notch in, groove down.” Circle on outside.

• Turn so that tall glass in front and small in back. Place on gasket.

• A & B knob columns-use one hand to assemble and place in prep area.

• B knobs in back.

• To prep apparatus, unscrew and flatten glass

Other reagents to be added immediately before pour gel:

• Ammonium Persulfate (APS) 10% (in bucket) add 160 μl to ea. tube (make fresh- weigh room .1 g/mL; .1 g and 1.0 mL water)
• Tetramethylethylenediamine (TEMED) - add 16 μl to each tube – on bench by mine.

• Add to set one (for 1st gel), and then dispense gel. Wait to add APS and TEMED to set two for companion gel.

• Functions of each:
  o APS- quenches oxygen radicals
  o TEMED- crosslinks the gel.

  Note: Once TEMED is added to the tubes, work quickly so the solution doesn’t gel in syringes.

Gel prep: Syringes with High and Low solutions:

• Syringes- there is one syringe for the low solution and one for the high solution

• Gradient Former-Gradient Delivery System -GDS (syringe-holder) is white. Marked for “low syringe.” Turn wheel to Left to dispense by placing bifurcation (“Y”) tubing (tubing with needle on end) in the syringe tubing and place attached needle between small and tall glass. Each side holds 15 mL; it’s set on 14.5. Switch to “high syringe” and dispense.

• Wash syringes w/ distilled water (and aspirate in and out.)

Then complete second set of High and Low: (160 μl APS and 16μl Temed) for companion gel. Do not add the APS and TEMED to second set of 50 mL falcon tubes until ready to dispense gel.

• Combs in – perpendicular then down (halfway.)

Electrical Universal Mutation Detection System:

• Heater on until 60C (shows actual)

• Let gel set for 1.5 hrs.

To Load gel w/sample : CORE

• Place glass/gels in CORE.
• Remove combs- indicate “good wells” with marker.
• Assemble .5 TAE (Tris-acetate-EDTA) buffer squirt bottle and razor blade to prepare wells.
• Cut comb part of gel with razor after lifting out comb.
• Squirt each well with .5 TAE.
• Wash Core (white part) with DI H₂O
• Pour 60°C running buffer on both sides (from electrophoresis tank)
• The red mark (circle) on the top of the Core goes to the rear on the right.
• Wash wells with 200 uL pipette (long tip) using running buffer.
• Load 15 uL PCR product (already has loading dye in it from when you ran PCR quality gels.)
• Best to wash four, then load four, repeat.
• Do a control and
• Use 100 bp ladder (generally 3-8 uL)
• Record A: gel and B: gel sample numbers.
• Place core in Electrophoresis tank with – UMDS (Universal Mutation Detection System) controls. UMDS: must reach 60°C. If using water bath, it must reach 60°C. Use 0.5 TAE buffer to fill tank to “run” line, not “fill line.”
• Set at 92-3 mAmperes/volts @15 hours (or 73 mAmperes @19 hours). Use similar volt hour totals in your calculations (1370-1390 volt hours.) Use EC-105 gel runner; not newer Biorad- will shut off.

Other options:
• Approx. 275- 280 mAmperes/volts @ 5 hours, or total equivalent volt hours.

After it gel has run:
• Place Electrophoresis control module in rack.
• Take CORE/gel out. Pull up on left first. Cut at diagonal on right top with slit spacer and cut longitudinally. (This also provides gel
orientation.) Carefully use squirt bottle of .5 TAE buffer to slide gel into Tupperware container. Gel very thin.

- Add 200-300 uL .5X TAE buffer (warm) from tank.
- Add 20-30 uL (depending on above; if added 200 TAE buffer, add 20 uL of stain.) Nucleic acid stain – SYBR GREEN ® (in freezer door Room A.)
- Place Tupperware, in cardboard box (White, Fisher- Room A refrigerator top) on Main room rocker for 30 minutes. Turn rocker on low. Tape box top and also tape box to rocker.

Room A:

- Blue rack- Rinse yellow frame with water.
- White plastic- glass plates, combs, spacers- wash w/ Liquinox detergent. Soak several hours/overnight.
- After gel has rocked, get plastic slide plate from Room A. Gently roll onto plastic slide plate. Roll onto Imager (using plastic slide plate). Blot. Visualize.

Denaturant Recipes:

7.5% acrylamide- 100% denaturant (urea/formamide) solution:

Beaker: 42 g urea*

40 mL formamide
18.75 mL 40% acrylamide
1 mL 50X TAE buffer

- Melt beaker contents on stir plate/stir bar.
- Using 100 mL graduated cylinder, add DI H2O to 100 mLs.

7.5% acrylamide- 0% denaturant (urea/formamide) solution

Beaker: 0 g urea

0 mL formamide
18.74 mL 40% acrylamide
1 mL 50X TAE buffer

(Same as above.)

* urea calculation: 7 M urea (m.w.= 60.06) x 7M = 420.42X 100/1000 = 42.042 g.
APPENDIX I

FOOD RECORD
# Food Record

Subject #___________________   Subject initials____________________

Date_______________________

**Day of Week** (Please circle one): Saturday / Sunday / Monday / Tuesday

**Study Period** (Please circle one): Baseline / Treatment

<table>
<thead>
<tr>
<th>Time of Day</th>
<th>Food/Drink consumed</th>
<th>Amount</th>
<th>Location (home/work/ restaurant)</th>
<th>Comments (Brand/How prepared)</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

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APPENDIX J

GASTROINTESTINAL TOLERANCE SYMPTOM RECORD
Gastrointestinal Tolerance
Symptom Record

Subject #___________________   Subject initials_______

Date: ______________________

Day of Week (Please circle one):    Saturday / Sunday / Monday / Tuesday

Study Period (Please circle one):  Baseline / Treatment

Please complete this before you go to bed for the night. Base it on any symptoms you may have had throughout the day. Place an “x” on the standardized 5 cm line below on the number corresponding to each possible symptom on the symptoms scale. See symptoms scale below. Thank you.

**Symptoms**

**Scale** (Key): 0 = none
1 = slight
2 = mild
3 = moderate
4 = moderately severe
5 = severe

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Number</th>
<th>Duration</th>
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</thead>
<tbody>
<tr>
<td>Abdominal Pain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bloating</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flatulence</td>
<td></td>
<td></td>
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<tr>
<td>Diarrhea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comments: ____________________________________________

If you place an “X” on a number other than zero, how long did the symptom last?
APPENDIX K

CHROMATOGRAMS
Fig. K.1 Subject #1: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Subject #3: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein

C: Glycitein **NOT DETECTABLE**
Fig. K.3 Subject #4: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.4  Subject #5: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.5 Subject #6: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.6  Subject #7: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.7 Subject #8: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.8 Subject #9: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.9  Subject #11: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.10 Subject #12: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.11 Subject #13: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.12 Subject #16: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.13  Subject #17: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.14  Subject #18: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.15 Subject #19: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.16 Subject #20: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.17 Subject #21: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.18  Subject #22: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein

A: Daidzein

B: Genistein

C: Glycitein
A: Daidzein

B: Genistein

C: Glycitein

Fig. K.19 Subject #23: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.20 Subject #24: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
Fig. K.21  Subject #25: Measurements of (A) Daidzein, (B) Genistein, and (C) Glycitein
APPENDIX L

INDIVIDUAL DGGE GELS
Diversity Index diff, 0.013; B-gal activity, 174

Diversity Index diff, 0.494; B-gal activity, -42

Diversity Index diff, 0.023; B-gal activity, -1.0

Diversity Index diff, 0.082; B-gal activity, 6

Diversity Index diff, 0.553; B-gal activity, 114

Diversity Index diff, 0.137; B-gal activity, -126

Diversity Index diff, 0.099; B-gal activity, 153

Diversity Index diff, 0.650; B-gal activity, 734
Diversity Index diff, -0.011; B-gal activity, -73

Pt on antibiotics - did not use data

Diversity Index diff, 0.089, B-gal activity, -105

Diversity Index diff, -0.220; B-gal activity, 43

Diversity Index diff, 0.117, B-gal activity, -53.3

Diversity Index diff, 0.032; B-gal activity, -50.5

Diversity Index diff, 0.821, B-gal activity, 449

Diversity Index diff, 0.461; B-gal activity, 16
Diversity Index diff, 0.880; B-gal activity, -618

Diversity Index diff, 0.157; B-gal activity, 101

Diversity Index diff, 0.375; B-gal activity, 272

Diversity Index diff, 0.469; B-gal activity, 907

Diversity Index diff, 0.084; B-gal activity, 19

Diversity Index diff, 0.102; B-gal activity, -162

Diversity Index diff, 0.018, B-gal activity, -20.8

Diversity Index diff, -0.305; B-gal activity, -268
APPENDIX M

ENTEROCYTE – FATE OF THE ISOFLAVONE AGLYCON AND GLUCOSIDE
Isoflavones (IFNs) are some of the active components of soy. For purposes of this presentation, there are 3 major forms of soy isoflavones (IFN) during digestion/absorption:

1. The IFN aglycone (nothing attached)
2. The IFN glucoside, also referred to as glycoside, (sugar attached)
3. The IFN glucuronide (“R” group attached. This is called a conjugated form)

Each one is discussed separately:

1. **The IFN-aglycone**: 
   - Can come in this form in soy (fermented) foods (about 20% of all soy ingested-eaten)
   - Is also the result of the glycoside or glucuronide groups being “cleaved off” by enzymes (β-glucosidase or β-glucuronidase.)
   - If the aglycone has nothing attached when eaten, a small amount can be absorbed through the stomach.
   - If the aglycone reaches the intestine, it is absorbed by the intestinal mucosa via what’s referred to as nonionic passive diffusion.
   - Can go to general circulation after absorption; more likely goes to the liver (via the portal vein) for conjugation via glucuronidation or sulfation. The liver has a box in it with UDP-GT, one of the conjugation enzymes.
   - The aglycone is now conjugated (glucuronidated or sulfated) and it is found this way in the general circulation (blood stream).

2 & 3. **The IFN-Glycoside and the IFN-Gluronide**:
   - The IFN-Glycoside is usually in this form when ingested. A glycoside is simply an IFN with a sugar attached.
   - Three potential locations of where the β-glucosidase enzyme can be:
     - in the small intestinal cell –the enterocyte (cytosolic β-glucosidase)
     - on the Brush border membrane (BBM-bound glucosidase)
     - bacterial glucosidase (in small intestine or large intestine)
IFN-glycoside goes through the enterocyte first, the sugar group is cleaved by the cytosolic β-glucosidase, but then the aglycone may be “glucuronidated” by the glucuronyltransferase (GT) enzyme in the enterocyte, which attaches an “R” group to it. (Glucuronidation occurs in both the liver and small intestine. Then, 2/3 of the IFN-glucuronide is shunted out of the enterocyte to basolateral membrane (BLM) to general circulation and 1/3 of the IFN-glucuronide is effluxed back to the intestinal lumen, where it is cleaved by a bacterial glucuronidase and absorbed as a simple aglycone via nonionic passive diffusion. It can then go to general circulation or to the liver. If the aglycone makes it out of the enterocyte without being glucuronidated it can be glucuronidated by the liver, or it can just go to general circulation. The ability of the bacteria in the lower part of the intestine (possibly distal part of the small intestine and most of the colon) to produce MORE β-glucosidase is the FOCUS of our research. Intestinal microflora convert aglycones to other isoflavone metabolites. The aglycones and selected metabolites can theoretically impart beneficial effects to tissues of the body. THE BLOOD STREAM ONLY HAS THE IFN-AGLYCONE FORM AND THE IFN-GLUCURONIDE FORM (OR SULFATE) FORMS IN IT. It is thought that sulfation could occur in the intestine, liver, or kidney.