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Theresa Anne Larkin
University of Wollongong

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SOY ISOFLAVONE BIOAVAILABILITY – EFFECTS OF PROBIOTIC AND PREBIOTIC CONSUMPTION AND OIL SUPPLEMENTATION

A thesis submitted in fulfilment of the requirements for the award of the degree

DOCTOR OF PHILOSOPHY

from

UNIVERSITY OF WOLLONGONG

by

THERESA ANNE LARKIN, BSc. (Hons) Advanced (Chemistry)

DEPARTMENT OF BIOMEDICAL SCIENCE

2005

CERTIFICATION

I, Theresa Anne Larkin, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Department of Biomedical Science, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Theresa Anne Larkin

11 April 2005

DEDICATION

This thesis is dedicated to my admirable grandparents. The unconditional love that Nan, Pa, Pop and Nana have shown to so many and their unique and amazing characters are inspirational and beautifully motivating.

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There are many people who I need to thank for their help during the course of my thesis. I have gained so much from, and thoroughly enjoyed, this experience.

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LIST OF ABBREVIATIONS

ANOVA.....	analysis of variance
ANOVA/RM.....	ANOVA with repeated measures
AUC.....	area under the curve
BMI.....	body mass index
CFU.....	colony forming units
C-max.....	maximum concentration
CVD.....	cardiovascular disease
D/G.....	daidzein: genistein ratio
DAID.....	daidzein
DHA.....	docosahexaenoic acid
ECD.....	electrochemical detector
EPA.....	eicosapentaenoic acid
EQL.....	equol
GEN.....	genistein
HPLC.....	high performance liquid chromatography
HRT.....	hormone replacement therapy
n-3.....	omega-3
ODMA.....	o-desmethylangolensin
OO.....	olive oil
PEP.....	p-ethyl phenol
PUFA.....	polyunsaturated fatty acids
SEM.....	standard error of the mean
RS.....	resistant starch
YC.....	control yoghurt
YP.....	probiotic yoghurt

ABSTRACT

Epidemiological evidence suggests a beneficial effect of soy consumption in relation to cardiovascular disease and in 1999, the U.S. Food and Drug Administration approved a health claim for the cholesterol-lowering effects of soy protein. However, this effect has not always been reproduced in subsequent soy dietary interventions, the results of which vary greatly. Soy beans are the richest dietary source of the isoflavones daidzein, genistein and glycitein, which have also been implicated with a role in lipid-lowering due to their antioxidant and estrogen receptor activities. After soy intake, there is large variability between individuals in isoflavone bioavailability. The isoflavones are present in soy as glycoside conjugates and after endogenous hydrolysis, the aglycones are absorbed, metabolised by liver and intestinal enzymes, distributed to tissues and excreted in urine. Endogenous isoflavone metabolites have been identified; of particular interest is the metabolism of daidzein to equol, as this has greater antioxidant and estrogen receptor activities than daidzein. Gut microflora is essential for isoflavone bioavailability and metabolism and can be affected by dietary modification. Differences in gut microflora composition may contribute to the large inter-individual variability in these processes, which in turn may contribute to variation in the lipid effects of soy consumption. Recently there has been much interest in the identification of dietary components that may enhance soy isoflavone bioavailability and it was the aim of this thesis to examine the effects of soy foods and other dietary components on soy isoflavone bioavailability and lipids. Four human studies were conducted and isoflavone bioavailability was determined based on plasma and urinary isoflavone levels. These samples were extracted with *Helix Pomatia* juice containing β -glucuronidases and sulphatases and the isoflavone aglycones were quantified by HPLC with electrochemical detection.

Resistant starch is a prebiotic and therefore has specific effects on gut microflora activity in the gastrointestinal tract and it was hypothesised that resistant starch intake may also affect isoflavone bioavailability. A pilot study with nine females (7 Australian and 2 Kenyan) was conducted to determine the acute and chronic effects of resistant starch

intake on soy isoflavone bioavailability. When resistant starch was consumed in the same meal as soy, plasma levels of daidzein and genistein were significantly reduced. However, daily resistant starch intake for 2 and 4 weeks prior to a soy meal resulted in a trend of increased mean daidzein excretion and of increased equol production in the two Kenyan subjects. It was concluded that resistant starch may enhance equol production, possibly dependent on gut microflora, genetics or habitual diet.

To determine whether there was an association between isoflavone bioavailability and lipid changes after soy intake, a soy dietary intervention was conducted in 23 hyperlipidemic men and postmenopausal women. Plasma and urinary daidzein and genistein levels were increased significantly after 6 weeks of soy milk and yoghurt intake. This treatment did not significantly affect lipids and there were no correlations between plasma or urinary isoflavone levels and lipid changes. However, in 8 subjects who produced equol in their plasma or urine, soy intake resulted in significant reductions in total and LDL cholesterol. This suggests that metabolism of daidzein to equol may be a determinant of the lipid-lowering effects of soy, contributing to this variation.

Based on the findings of the first two studies, a dietary combination of soy (cereal and milk) with either a probiotic or a prebiotic was proposed for further examination of the effects of resistant starch and equol production on isoflavone bioavailability and lipid levels. In a study of crossover design with 5-week dietary periods, soy consumption was compared with intake of soy plus either probiotic yoghurt or resistant starch-enriched bread for the effects on plasma and urinary isoflavone levels after a test soy meal in 31 hyperlipidemic men and postmenopausal women. Soy intake significantly increased circulating plasma daidzein and genistein levels, but did not affect plasma or urinary isoflavones after the test soy meal. There were no additional significant effects of either probiotic or prebiotic treatments; however, there was a trend for increased circulating plasma daidzein and genistein with probiotic treatment and for increased plasma daidzein and genistein 24 hours after the test soy meal with prebiotic treatment. Probiotic or prebiotic treatment did not induce or increase equol production, though there was a trend for increased plasma equol in “equol-positive” subjects (n = 12) after probiotic treatment.

The lack of any overall significant effects on isoflavone bioavailability with either probiotic or prebiotic treatment suggests that even if gut microflora was modified, this was not favourable for isoflavone bioavailability or equol production and thus it appears that other inherent features may determine these processes. Total cholesterol was significantly decreased with soy plus probiotic or prebiotic intake ($-4.7 \pm 2.0\%$ and $-5.5 \pm 1.6\%$ respectively) and LDL cholesterol was significantly decreased with soy intake and with prebiotic treatment ($-4.1 \pm 2.1\%$ and $-7.3 \pm 2.2\%$ respectively). This suggests that even in the absence of effects on isoflavone bioavailability, there was synergistic action between soy and probiotic or prebiotic intake for lipid-lowering effects and thus combination of these dietary components may be useful in lipid management.

For further examination of potential lipid-lowering effects of soy in synergy with other dietary components, it was hypothesised that a novel combination of soy with DHA-rich oil may also affect isoflavone bioavailability and result in a more positive lipid profile than supplementation with either component alone. DHA supplementation has strong triglyceride lowering effects, but it often also results in elevated LDL cholesterol, whereas conversely, a reduction in LDL is the most commonly reported lipid effect of dietary soy intake. In a crossover study with 35 hyperlipidemic men and postmenopausal women, plasma and urinary isoflavones were significantly increased after 6 weeks of soy (cereal) intake, but there were no significant effects of DHA-rich oil supplementation. Soy intake did not result in any significant lipid effects; however DHA supplementation resulted in a significant increase in HDL and decrease in triglycerides, independent of concurrent soy intake. In addition, there was an influence of the combination of DHA and soy compared with DHA alone for total and LDL cholesterol. While total and LDL cholesterol were increased with DHA supplementation alone, significantly for LDL, these increases were somewhat attenuated with concurrent soy intake. This suggests the potential for a combination of soy and n-3 fatty acids in producing lipid effects protective in relation to cardiovascular disease.

When the latter three studies were compared, a relation between the daidzein and genistein levels of food with the ratios of these isoflavones in plasma and urine was

evident, however some effects of soy food matrix were observed. Soy milk intake resulted in greater genistein bioavailability than daidzein compared to their relative amounts in the soy milk, while resistant starch intake appeared to increase daidzein excretion more than genistein. Furthermore, there was a particular finding of high occurrence of equol in plasma in the third study examining the combination of soy with oil; this was suggested to be due to the higher proportions of daidzein and glycitein than genistein in the soy germ product ingredient of the cereal.

Overall, there appears to be the potential for probiotic and prebiotic foods and the soy matrix isoflavone composition to influence soy isoflavone bioavailability. These findings are important in relation to physiological activities of soy foods as the isoflavones differ in their bioactivity and require further investigation. Further, in relation to lipid effects of soy consumption, baseline levels of total and LDL cholesterol were both significantly inversely correlated with subsequent lipid changes with soy intake. In addition, there were beneficial additive hypocholesterolemic effects of soy with probiotic and prebiotic foods and with DHA-rich oil supplementation. In conclusion, the bioavailability of isoflavones from soy is affected by other dietary components and the soy matrix in which they are contained. This did not appear to influence lipid effects which were modest and only significant when soy was consumed concurrently with other dietary components also known to produce beneficial effects. The latter finding has application in the development of functional foods for those with elevated lipids.

PUBLICATIONS ARISING FROM THIS THESIS

Larkin TA, Astheimer L, Price WE (2001) Health benefits of dietary phytoestrogens. *Agro Food Industry Hi-Tech* **12**, 19 – 21.

Larkin TA, Astheimer L, Price WE (2000) Analysis of Phytoestrogens in foods and their bioavailability. *Agro Food Industry Hi-Tech* **11**, 24-27.

Meyer BJ, Larkin TA, Owen AO, Astheimer LB, Tapsell LC, Howe PRC (2004) Limited Lipid Lowering Effects of Regular Consumption of Whole Soy Bean Foods. *Annals of Nutrition and Metabolism* **48**, 67-78.

Meyer BJ, Larkin TA, Owen AJ, Astheimer LB, Tapsell L, Howe PRC (2002) The hypocholesterolaemic effect of chronic soy consumption may be linked to equol consumption. In 'Soy Health 2002: Clinical Evidence Dietetic Applications' pp. 53-61. (Garant)

Owen AJ, Larkin TA, Ridges LA, Meyer B, Astheimer L (submitted) A characterisation of lipoprotein phytoestrogen content

Conference Poster Presentations

- Larkin T, Price WE, Astheimer L (2003) Effect of a combination of soy with a prebiotic or probiotic on isoflavone bioavailability. *1st International Conference on Polyphenols and Health*. Vichy, France
- Larkin T, Price WE, Astheimer L (2003) Effect of soy consumption and equol production on cholesterol hypercholesterolemic patients. *1st International Conference on Polyphenols and Health*. Vichy, France

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- Ridges LA, Larkin TA, Martin G, Meyer BJ, Astheimer L, Howe P (2004) Dietary soy isoflavones offer protection against LDL elevation in mildly hyperlipidemic people consuming a DHA-rich oil supplement. *2nd Australian Health and Medical Research Council Conference*. Sydney, Australia

CHAPTER 1

LITERATURE REVIEW

1.1 SCOPE OF THESIS

This thesis was undertaken in the broad research area of soy foods and their relevance to human health. More specifically, the research was focused on the examination of the effects of other dietary factors on isoflavone bioavailability as determined by plasma and urinary levels. In addition, lipid measures were also included, being more clinically relevant and topical, particularly in relation to isoflavone bioavailability. Thus, this literature review will provide information pertinent to the fate of isoflavones in humans after soy intake, including their absorption and metabolism and factors affecting these processes. The literature related to the health benefits of isoflavones in humans will also be discussed to provide a context for the direction of the research included in this thesis.

1.2 HEALTH BENEFITS OF SOY CONSUMPTION – EPIDEMIOLOGICAL EVIDENCE

Diets low in fat and high in fibre and complex carbohydrates from grains, fruits, vegetables and legumes have long been associated with a reduced incidence of cardiovascular disease (CVD) and many cancers (Slavin *et al.*, 1997). In contrast to such diets, including those of Asian origins, the typical Western diet is associated with an increased risk of the aforementioned chronic diseases (Adlercreutz, 1990). Apart from the differences in fat and dietary fibre content, other constituents of diets high in legumes and grains have been implicated as having health beneficial effects, including phytoestrogens (Adlercreutz, 1984; Tham *et al.*, 1998; Thompson, 1994).

Phytoestrogens are plant compounds with hormonal activity (Adlercreutz and Mazur, 1997) and structural similarity to endogenous estrogens and include isoflavones from soy and other legumes, lignans from linseed and whole grains, and coumestans from clover.

Among Asian populations with a high intake of soy, epidemiological evidence has demonstrated a lower incidence of cardiovascular disease (Adlercreutz, 1990), hormone-dependent cancers of the breast and prostate (Yu *et al.*, 1991), colon cancer (Rose *et al.*, 1986), menopausal symptoms (Clarkson, 2000) and osteoporosis (Adlercreutz *et al.*, 1992). These effects have been extensively reviewed and will not be reiterated here (Adlercreutz, 1995; Bingham *et al.*, 1998; Cassidy *et al.*, 2000; Knight and Eden, 1996; Kurzer and Xu, 1997; Messina *et al.*, 1994; Murkies *et al.*, 1998; Setchell and Cassidy, 1999; Tham *et al.*, 1998; Wu *et al.*, 1998). However, it should be noted that there is also a strong association between dietary fat intake and cancers of the breast, prostate and colon (Rose *et al.*, 1986). Although native Asian women and men have the lowest rates of breast (Henderson and Bernstein, 1991) and prostate cancer (Giovannucci, 1995; Ross *et al.*, 1995; Yu *et al.*, 1991), respectively, migration to Western countries and the adoption of a more Western diet, increases the incidence of these cancers amongst migrant Asians to an occurrence similar to Western populations (Whittemore *et al.*, 1995; Ziegler *et al.*, 1993). Similarly, while a cross-sectional study in Japan revealed an inverse association between soy intake and serum total cholesterol concentration (Nagata *et al.*, 1998), an increase in the incidence of cardiovascular disease is reported for migrant Japanese (Kim *et al.*, 1998b). Thus diet, and in particular soy and its isoflavones, have been implicated as affording some protection against the development of these hormone dependent cancers and cardiovascular disease.

1.3 SOY – FOOD SOURCES AND ISOFLAVONE COMPOSITIONS

1.3.1 Isoflavone synthesis and distribution

Isoflavones are not widely distributed in plants, occurring almost exclusively in legumes (Coward *et al.*, 1993). Soybeans are the richest source of the plant precursors of the endogenous isoflavones, daidzein (4',7-dihydroxyisoflavone), genistein (4',5,7-trihydroxyisoflavone) and glycitein (4',7-dihydroxy-6-methoxyisoflavone) (Figure 1.1). Isoflavones are synthesised as part of the phenyl-propanoid pathway, which has multiple branches common to legume and non-legume plants and from which other flavonoids are also synthesised (Hollman, 2001; Parr and Bolwell, 2000; Yu *et al.*, 2000). Their occurrence is limited because isoflavone synthase, the enzyme required to convert their flavanone precursors, is unique to legumes and only a few other species (Rolfe, 1988; Yu *et al.*, 2000). Isoflavone synthesis is developmentally and tissue-specifically regulated and may be induced by environmental stresses, particularly as the natural roles for isoflavones are in plant-microbial interactions, including disease resistance (Ebel, 1986; Yu *et al.*, 2000). Formononetin and biochanin A, the 4'-O-methoxylated isoflavone derivatives and precursors to daidzein and genistein, respectively (Figure 1.1), occur in alfalfa and clover seeds and sprouts, and in chick peas, garbanzo beans, black bean seeds and some pulses (Franke *et al.*, 1994; King *et al.*, 1994; Murphy *et al.*, 1999; Shoff *et al.*, 1998; Wang and Murphy, 1994a).

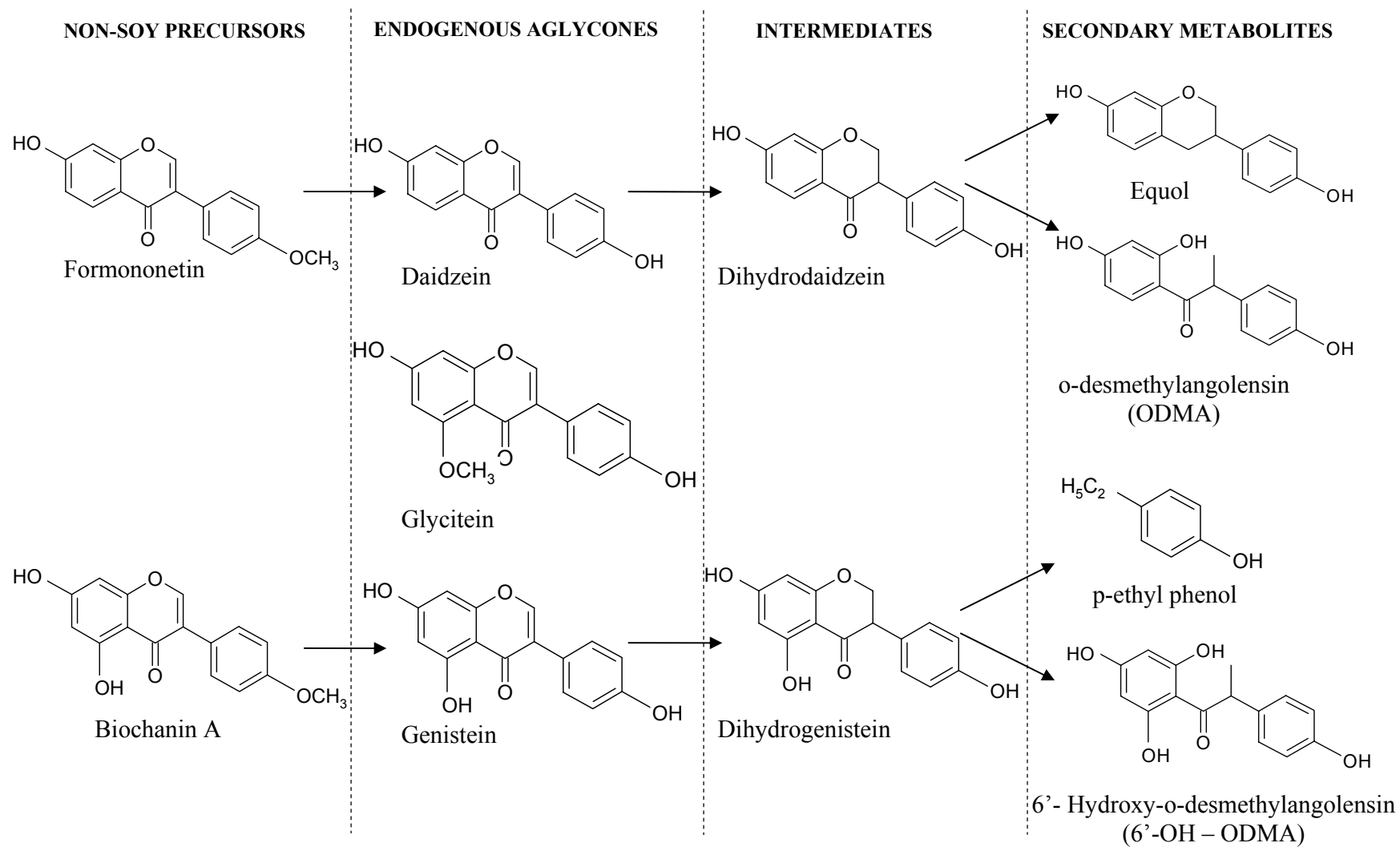


Figure 1.1 Isoflavone structures

1.3.2 Soybean composition

Soybeans are composed of the fleshy cotyledons, the part of the seed that forms the first plant leaves; the hypocotyl, the part of the axis of the plant embryo below the cotyledon and the hull, the dry outer covering of the seed (Price and Fenwick, 1985). The isoflavones are present in four isomeric forms in soybeans: aglycones and three glucoside conjugates, including the β -, acetyl- and malonyl-glucoside- conjugates (Wang and Murphy, 1994b; Xu *et al.*, 2000; Figure 1.2). The aglycones have no sugar residue attached, whereas the glucoside conjugates have a sugar group attached at the position 7 of the A ring. In soybeans, the majority of isoflavones are concentrated in the hypocotyl, where daidzein, glycitein and their respective conjugates account for more than 95% of total isoflavones (Price and Fenwick, 1985). Glycitein and its three derivatives occur exclusively in the hypocotyl (Wang and Murphy, 1994a), while genistein is found both in the hypocotyl and cotyledon, predominating in the latter (Erdman Jr *et al.*, 2004; Price and Fenwick, 1985). An early analysis of soybean fractions revealed total isoflavone concentrations of 1405 – 1750 mg/100 g in the hypocotyl, 319-808 mg/100 g in the cotyledon and 10 – 20 mg/100 g in the hull (Price and Fenwick, 1985).

Total isoflavone content of soybeans varies widely and can be affected by crop year, soil conditions, local climate, genetics and stage of maturity (Franke *et al.*, 1994; Simonne *et al.*, 2000; Wang and Murphy, 1994a). In addition, subsequent processing and storage conditions such as duration, light and temperature will also affect isoflavone yield (Eisen *et al.*, 2003; Wang *et al.*, 1990; Wang and Murphy, 1994b). Amongst different varieties, total isoflavone levels have been reported to range from 0.1 to 5 mg per gram of soybean (Barnes *et al.*, 1994; Coward *et al.*, 1993; Franke *et al.*, 1995; Simonne *et al.*, 2000; Wang and Murphy, 1994a). Genistein is generally present at higher levels than daidzein and glycitein in soybeans and most soy-derived foods (Franke *et al.*, 1995; Setchell *et al.*, 2001), up to 1600 $\mu\text{g/g}$ compared with up to 1100 $\mu\text{g/g}$ daidzein and 600 $\mu\text{g/g}$ glycitein (Franke *et al.*, 1998; Simonne *et al.*, 2000; Wang and Murphy, 1994a). The variability in soy bean isoflavone composition is also reflected in other soy-derived foods, including

soy milk from different regions of the U.S, for which the total isoflavone concentrations varied by up to approximately 70% (Murphy *et al.*, 1999).

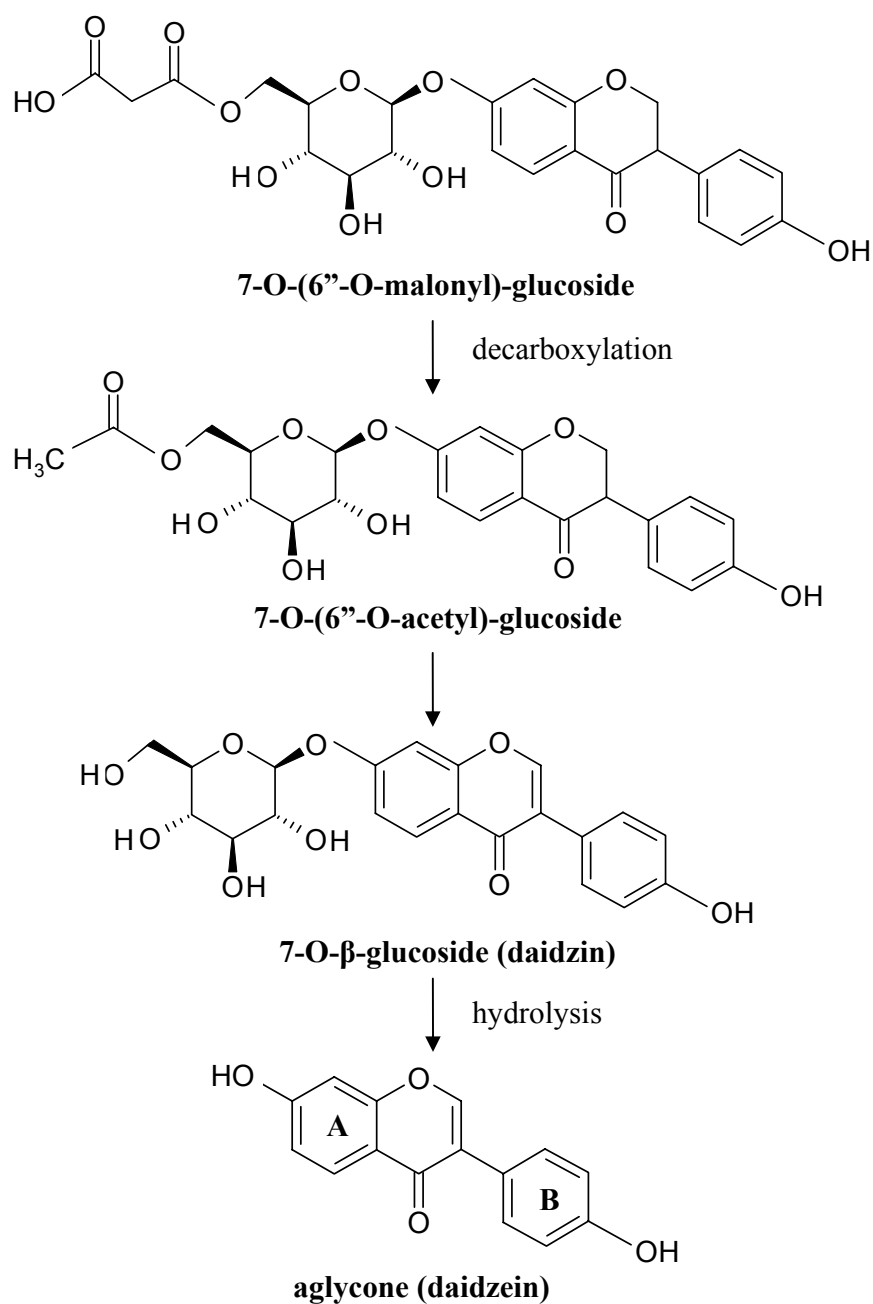


Figure 1.2 The four different isomers of daidzein in soy foods. Analogous structures of genistein and glycitein are also present in soybeans.

1.3.3 Dietary soy intakes and isoflavone content

In traditional Asian diets, soy is consumed in many forms including soybeans, soybean sprouts, toasted soy protein flours, soy milk, tofu and fermented soybean products such as miso, tempeh, soybean paste, natto and soy sauce (Coward *et al.*, 1993; Wang and Murphy, 1996). Soybean consumption per individual in most Asian countries is reported to be about 35 g per day (Coward *et al.*, 1993), and is highest among native Japanese, with average intakes of 70 g soybean per day (Watanabe *et al.*, 1998). This equates to a daily intake of between 25 and 100 mg total isoflavones (Coward *et al.*, 1993; Messina, 1999; Setchell *et al.*, 2001) and up to 300 mg in the Japanese diet and between 8 and 12 g soy protein (Erdman Jr *et al.*, 2004). In Western countries, soy intake is more commonly in the form of soybean protein products including flours, grits, isolates, concentrates and textured soy proteins (Wang and Murphy, 1996) and the average daily exposure to isoflavones is less than 1 mg (Adlercreutz *et al.*, 1991b; Messina *et al.*, 1994; Wei *et al.*, 1995). Greater soy intake is reflected in plasma and urinary isoflavone concentrations, which are higher in Japanese men and women than Western populations (Adlercreutz *et al.*, 1991b) and are also higher in vegetarians than omnivorous subjects (Adlercreutz *et al.*, 1993).

1.3.4 Isoflavone composition of soybean-derived foods

In whole soybeans, the isoflavones occur mostly as 6"-O-malonylglucoside conjugates, with the β -glucosides (daidzin and genistin) being the second most abundant isoflavone derivatives (Coward *et al.*, 1993). In a study that examined eight American varieties of soybeans, 6"-O-malonylgenistin was the major isoflavone constituent, followed by genistin, 6"-O-malonyldaidzin and daidzin respectively, with these four derivatives comprising 83 – 93% of the total isoflavone content (Wang and Murphy, 1994a). In whole soybeans and other soy protein products, 97 – 98% of the isoflavones are present as their esterified conjugates (Wang and Murphy, 1994b); however, the glucoside

composition of soybean-derived foods varies, being determined by processing conditions (Coward *et al.*, 1998).

Hot or acidic extraction procedures cause decarboxylation of the 6"-O-malonylglucoside conjugates in whole soybeans to produce the 6"-O-acetylglucosides conjugates, which can further undergo ester hydrolysis to form the β -glucosides (Barnes *et al.*, 1994; Coward *et al.*, 1993; Murphy *et al.*, 1999; Simonne *et al.*, 2000). While this does not necessarily change the total amount of isoflavones extracted, the ratio of conjugated forms varies between soy foods (Coward *et al.*, 1998). Soy milk and tofu are produced from soybeans via hot aqueous extraction which results almost entirely in the formation of β -glucosides (Barnes *et al.*, 1994; Coward *et al.*, 1998; Eisen *et al.*, 2003; King and Bursill, 1998). In fermented soy products, unconjugated aglycones, resulting from the action of the β -glucosidases of the fermentation organisms, are the predominant chemical forms (Coward *et al.*, 1993; Fukutake *et al.*, 1996; Murphy *et al.*, 1999). Compared with whole soybeans, soy flour and soybean seeds generally contain slightly less total isoflavone content (Franke *et al.*, 1994; Franke *et al.*, 1995), while soy nuts, which are harvested at a later time and dried, can have higher levels (Fukutake *et al.*, 1996). Soy germ products contain higher concentrations of daidzein and glycitein than genistein due to the lesser levels of the latter in the hypocotyl (Zhang *et al.*, 1999).

It appears that the isoflavones are associated with the soluble components of soybean, probably soluble proteins (Wang and Murphy, 1996) and therefore, high protein soy ingredients contain similar isoflavone concentrations compared with unprocessed soybeans (Eisen *et al.*, 2003). Soy protein isolate produced from hot water extraction of soy flour retains most of the isoflavones, probably reflecting their strong protein binding and low aqueous solubility, and the same pattern of conjugation as soybean, (Coward *et al.*, 1993). Soy protein can have a total isoflavone content of up to 2.7 mg/g, which is comparable to that of soy flour and many Asian soybean products (Coward *et al.*, 1993); however, it varies as to whether daidzein or genistein is the most concentrated isoflavone (Barnes *et al.*, 1994; Franke *et al.*, 1998). In contrast, ethanol extraction of soy flour to

produce soy protein concentrate removes most of the isoflavones (Coward *et al.*, 1993; Coward *et al.*, 1998).

1.4 MECHANISMS OF ACTION OF SOY ISOFLAVONES IN HUMANS

1.4.1 Isoflavone structure in relation to physiological activity

Isoflavones have both estrogenic activity and antioxidant capacity, related to their structural similarity to 17 β -estradiol (Figure 1.3). The presence of the phenolic ring, the position of and distance between the two hydroxyl groups and the diaryl ring structure, are considered prerequisite for estrogenic activity (Cassidy, 1996; Cassidy *et al.*, 2000; Miksicek, 1995; Tham *et al.*, 1998). Similarly, the antioxidant capacity of the isoflavones is closely associated with the presence of the hydroxyl groups and the position of the aromatic ring (Tham *et al.*, 1998). These activities are relevant to the potential roles of isoflavones in reducing cancer and cardiovascular disease risk, as free radicals, as well as high endogenous hormone levels have been linked with the development of these conditions (Zheng and Zhu, 1999).

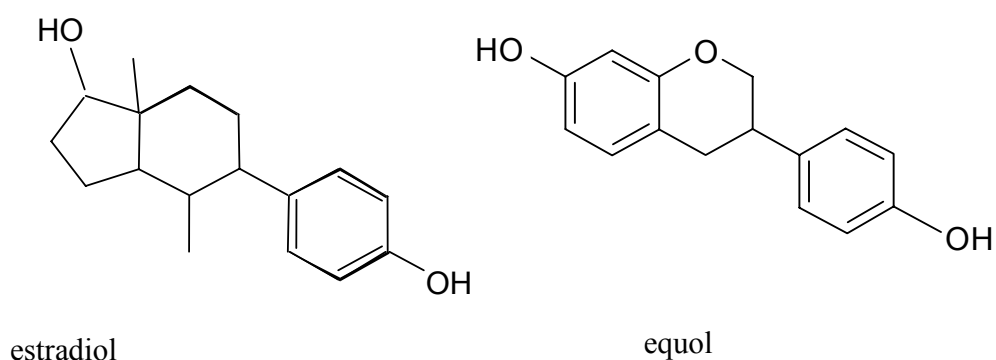


Figure 1.3 Structural similarity between estradiol and the isoflavone metabolite equol.

1.4.2 Isoflavone actions via the estrogen receptor

It is well established that the isoflavones are able to bind to estrogen receptors (ER) and elicit either a weak estrogenic (agonistic) or anti-estrogenic (antagonistic) effect, depending on the levels of endogenous estrogens present (Baghurst, 1997; Wang and Kurzer, 1998). In the presence of estradiol (e.g. premenopausal women), ER-bound isoflavones will reduce receptor activation compared with estradiol and behave as an antagonist or anti-estrogen (Baghurst, 1997; Persky *et al.*, 2002; Sathyamoorthy and Wang, 1997). However, when estrogen is either absent or only present in very low concentrations (e.g. men or postmenopausal women) then the isoflavones may partially activate the receptor, producing a weak agonistic response (Anderson *et al.*, 1999; Baghurst, 1997; Persky *et al.*, 2002).

The subsequent effects of estrogen receptor binding of isoflavones also largely depend on the tissue and receptor type (Kuiper *et al.*, 1998; Setchell and Cassidy, 1999). The two estrogen receptor subtypes (ER- α and ER- β) are differentially distributed in the body, with tissues of the breast, uterus, ovary and vascular epithelium expressing both subtypes, while prostate, lung, bone and brain only express ER- β (Setchell and Cassidy, 1999). Isoflavones have a much higher affinity for ER- β than ER- α (Kuiper *et al.*, 1997; Nikov *et al.*, 2000), thus tissues with higher expression of ER- β might be more responsive to isoflavones (Anderson *et al.*, 1999). Genistein and equol bind to ER- β with similar affinity as 17 β -estradiol, although their estrogenic activity is in the order of 1000-fold less (Nikov *et al.*, 2000; Sathyamoorthy and Wang, 1997). However, the levels of isoflavones in plasma can be 1000 times higher than estrogen levels in women (Ishimi *et al.*, 1999; Setchell and Adlercreutz, 1998). The differential distribution of the estrogen receptors also means that an estrogen agonist could produce different effects in various tissues, and conversely, that a particular tissue could respond differently to various estrogen agonists (Anderson *et al.*, 1999). For example in female mice, genistein has been shown to prevent bone loss caused by estrogen deficiency but without substantial negative estrogenic effects on the uterus (Ishimi *et al.*, 1999). Thus, the

estrogen receptor activity of isoflavones may play a major role in their effects against cancers of tissues that express estrogen receptors (Cassidy, 1996) and in the alleviation of menopausal symptoms (Persky *et al.*, 2002; Wilcox *et al.*, 1990).

1.4.3 Antioxidant activities of soy isoflavones

Due to their phenolic nature, isoflavones show good antioxidant activity in a number of *in vitro* chemical and biological systems (Mitchell *et al.*, 1998; Zheng and Zhu, 1999) in both aqueous and lipophilic phases (Harper *et al.*, 1999; Ruiz-Larrea *et al.*, 1997). The free 4'-OH of isoflavones (Figure 1.1) makes them good free radical scavengers (Mitchell *et al.*, 1998), but they can also act as proton donors (Arora *et al.*, 1998), chelate iron and bind to DNA (Harper *et al.*, 1999). The *in vitro* activities of genistein and daidzein have been observed in the micromolar range (Record *et al.*, 1995) and they appear to act synergistically (Vedavanam *et al.*, 1999). Genistein can inhibit the formation of 8-hydroxy-2'-deoxyguanosine, an oxidation product that is increased in cancerous tissues and may be carcinogenic itself (Zheng and Zhu, 1999) and soy supplementation has also decreased oxidative DNA damage in healthy volunteers after 4 weeks intake (Mitchell and Collins, 1999). The inhibition of lipid peroxidation, particularly of LDL, by isoflavones may be an important mechanism by which they positively influence lipid profiles.

1.5 EFFECTS OF SOY INTAKE ON DISEASE RISK FACTORS

1.5.1 Clinical evidence for beneficial effects of soy in relation to cardiovascular disease

In 1999, the U.S. Food and Drug Administration approved a health claim for the cholesterol-lowering effects of soy protein, based on a recommended intake of 25 g/day. This health claim followed the publication of a meta-analysis of 38 clinical trials that reported significant decreases in total and LDL cholesterol and triglycerides with soy

protein intake compared with animal protein consumption (Anderson *et al.*, 1995). However, there is much variation within, and controversy between, results of clinical trials using dietary soy or soy protein and isoflavone supplementation in investigating lipid and other cardiovascular effects (Lichtenstein, 1998). This was highlighted in a recent meta-analysis of isoflavone interventions in the form of soy proteins or other soy diets, in which the results were inconclusive (Yeung and Yu, 2003). It is hard to compare many of the studies directly however, as endogenous hormone levels (Potter *et al.*, 1998) and baseline lipids of subjects (Gardner *et al.*, 2001) as well as the dietary source of soy, isoflavone concentration (Steinberg *et al.*, 2003), intervention duration and study design (Merz-Demlow *et al.*, 2000) all may impact on the results.

For improvements in lipid levels, it appears that the combination of soy protein and isoflavones may be more beneficial than either component alone (Potter, 1998; Steinberg *et al.*, 2003). Isoflavone supplements alone generally do not lower lipids (Hodgson *et al.*, 1998; Nestel *et al.*, 1997) as confirmed in a recent meta-analysis (Yeung and Yu, 2003). However, others have reported that in studies using soy protein, the isoflavones are necessary and result in dose-dependent lipid-lowering (Crouse *et al.*, 1999; Merz-Demlow *et al.*, 2000; Wangen *et al.*, 2001). Although the recent meta-analysis by Weggemans and Trautwein (2003) demonstrated a 4% reduction in LDL cholesterol with daily consumption of 36 g of soy protein in combination with 52 mg of soy-associated isoflavones, neither the isoflavone content nor soy protein amount had a significant influence on lipid changes. Thus, the soy protein matrix of isoflavones and intact soy protein (Gardner *et al.*, 2001; Steinberg *et al.*, 2003) has been implicated in the lipid-lowering effects of soy. Although the role of the individual soy components in their influence on lipids has not been fully elucidated, soy protein may affect hepatic metabolism of cholesterol or lipoproteins (Potter, 1998) or up-regulate LDL receptors (Anderson, 2003), while the isoflavones may act via their estrogenic and antioxidant activities.

The increased risk of CVD in postmenopausal women is largely attributed to decreases in endogenous estrogens associated with menopause (Clarkson *et al.*, 1995). The

administration of estrogen, such as the use of hormone replacement therapy (HRT) can decrease LDL and increase HDL cholesterol (Matthews *et al.*, 1989) and a similar role may exist for isoflavones due to their estrogenic activity. In postmenopausal women, estrogenic effects of soy supplementation include increases in HDL cholesterol (Persky *et al.*, 2002; Potter *et al.*, 1998), reductions in total and LDL cholesterol (Gardner *et al.*, 2001; Steinberg *et al.*, 2003; Wangen *et al.*, 2001), decreases in triglycerides (Cuevas *et al.*, 2003) and an increase in LDL receptor activity (Baum *et al.*, 1998; Tikkanen *et al.*, 1998). Genistein also enhances TGF- β which may produce cardioprotective and positive bone effects (Kim *et al.*, 1998b).

Oxidative modification of LDL is considered the first step in the development of atherosclerosis, allowing LDL uptake by macrophages in the arterial wall (Tikkanen *et al.*, 1998). Thus, LDL oxidation has been a focus for determining the antioxidant effects of isoflavones. *In vitro* evidence (Meng *et al.*, 1999) as well as *ex vivo* results from isolated human LDL (Jenkins *et al.*, 2000) and *in vivo* markers of oxidation have all shown decreased lipoprotein oxidation after soy consumption (Tikkanen *et al.*, 1998; Wiseman *et al.*, 2000). It has also been demonstrated that esterified isoflavones can be incorporated into LDL particles, increasing their *in vitro* oxidation resistance (Meng *et al.*, 1999). Further, both aglycone isoflavones (Owen *et al.*, in press) and glucoside conjugates can associate with lipoproteins and inhibit lipid peroxidation (Arora *et al.*, 2000).

The antioxidant activity of isoflavones may also be responsible for other cardioprotective effects including a reduction in hypertension. Isoflavones appear to protect against nitric oxide inactivation, this being one of the most potent vasodilators released by endothelial cells (Cuevas *et al.*, 2003). This mechanism may explain results indicating the alleviation of hypertension with soy intake via improved arterial compliance (Nestel *et al.*, 1997), dilation (Cuevas *et al.*, 2003) or a decrease in blood pressure (Crouse *et al.*, 1999). In addition, genistein is also a potent inhibitor of tyrosine kinase activity which is involved in smooth muscle cell contraction. However, not all studies have reported positive effects of soy in relation to hypertension (Hodgson *et al.*, 1999).

1.5.2 Effects of soy on breast and prostate cancer

Soy intake has also been associated with reduced incidence of the hormone-dependent cancers of the breast and prostate (Yu *et al.*, 1991). Although fewer controlled, prospective studies have been conducted in this area compared with cardiovascular research, the estrogenic, antioxidant and anti-cancer activities of the isoflavones have all been implicated in reducing the occurrence or development of these hormone-dependent cancers. In epidemiological and cross-sectional studies, a substantial reduction in breast cancer risk has been reported among women with increased soy intake or high excretion of isoflavones (Ingram *et al.*, 1997; Stephens, 1997a; Yamamoto *et al.*, 2003; Zheng *et al.*, 1999). Further, soy intake has been associated with reduced mammographic density, an intermediate marker of breast cancer risk (Frankenfeld *et al.*, 2004; Jakes *et al.*, 2002). There is also much *in vitro* evidence supporting inhibition of breast cancer development by isoflavones in human cancer cell lines (Constantinou *et al.*, 1998; Martin *et al.*, 1978; Pagliacci *et al.*, 1994; So *et al.*, 1997; Zava and Duwe, 1997) and in rats (Constantinou *et al.*, 1998; Lamartiniere *et al.*, 1998). In premenopausal women, soy supplementation has anti-estrogenic effects related to a decreased risk of breast cancer including a decrease in luteinizing hormone (Duncan *et al.*, 1999) and an increase in the length of the follicular phase of the menstrual cycle (Cassidy *et al.*, 1994; Smith *et al.*, 1997); although others found no effect on hormone levels or menstrual cycle length (Martini *et al.*, 1999).

Case-control studies have also reported an inverse relationship between soy intake and risk or incidence of prostate cancer (Jacobsen *et al.*, 1998; Severson *et al.*, 1989). In prostate cancer patients, isoflavone supplementation can reduce prostate-specific antigen (PSA), a marker of prostate cancer progression (Dalais *et al.*, 2004; Hussain *et al.*, 2003; Kumar *et al.*, 2004) and has resulted in apoptosis of adenocarcinoma of the prostate (Stephens, 1997b; Jarred *et al.*, 2002). Soy intake has also been shown to inhibit prostate cancer growth and the activity of 5 α -reductase, an enzyme which converts testosterone to a more potent androgen related to prostate cancer development (Barnes and Peterson, 1995; Kim *et al.*, 1998b). Numerous other activities of isoflavones have also been reported in relation to their anti-cancer effects (Barnes and Peterson, 1995; Constantinou

and Huberman, 1995; Lian *et al.*, 1999; Record *et al.*, 1995; Stephens, 1997a; Wei *et al.*, 1995).

Breast and prostate cancer are often associated with increased serum levels of free estrogens and androgens respectively (Gann *et al.*, 1996; Stephens, 1997a). Sex hormone-binding globulin (SHBG) transports these steroids in blood, decreasing their bioavailability, and has been positively correlated with urinary equol (Adlercreutz *et al.*, 1987) and was significantly higher in vegetarians among adult men (Belanger *et al.*, 1989). *In vitro*, isoflavones increase SHBG in human hepatoma cell lines (Loukovaara *et al.*, 1995) and *in vivo*, increases in plasma SHBG occur via increased hepatic synthesis (Adlercreutz *et al.*, 1987); however, soy supplementation studies have not produced clear results. While a few clinical trials with soy or isoflavone supplementation in men and women have shown modest increases (Persky *et al.*, 2002; Pino *et al.*, 2000; Smith *et al.*, 1997), others have found no change (Cassidy *et al.*, 1994; Kumar *et al.*, 2004; Martini *et al.*, 1999; Teede *et al.*, 2004). SHBG can bind and potentially transport isoflavones to tissues (Jury *et al.*, 2000) as evident by the presence of SHBG in breast cancer cells (Martin *et al.*, 1996).

1.5.3 Variability in health outcome measures with soy supplementation

Many soy dietary intervention studies report large variability in outcome measures and there has been limited reproducibility between studies. Mechanistically, the actions of soy and its specific components responsible for the health effects in chronic disease states, such as cardiovascular disease and cancer, have not yet been fully elucidated, which adds to the difficulty in explaining inter-individual differences. Inherent characteristics, such as genetics and endogenous hormones, as well as environmental factors will influence how individuals respond to particular dietary treatments. In relation to soy-derived foods, the large inter-individual variability in clinical and physiological effects may depend greatly on isoflavone bioavailability and the soy matrix in which they are contained.

1.6 ISOFLAVONE BIOAVAILABILITY

1.6.1 Importance of measures of bioavailability

The bioavailability of an ingested substance is defined as the amount that is absorbed and reaches the systemic circulation from where it can be distributed to tissues for physiological effects. A measure of the bioavailability of isoflavones is important in assessing their potential health benefits and may assist in the interpretation of the high variability of results in clinical trials. Bioavailability is not entirely indicated by the extent of plasma absorption, as the pharmacokinetics of absorption, distribution, metabolism (bioconversion in the gut and biotransformation in the liver) and elimination all contribute to the bioavailability and subsequent effectiveness of the isoflavones (Rowland *et al.*, 2003; Wiseman, 1999). However, most pharmacokinetic and bioavailability studies of isoflavones in humans are limited to plasma and urinary concentrations of specific isoflavones and their metabolites due to the ethical and practical difficulties of tissue measurements. This does not provide a comprehensive understanding of bioavailability and distribution, as plasma isoflavone concentrations simply represent the balance between absorption, distribution and urinary and biliary excretion (King, 1998). In addition, the use of only plasma and urinary measurements of isoflavones does not differentiate between the contributions of the intestine, liver or other organs in isoflavone metabolism (Liu and Hu, 2002). A representation of the processes involved in isoflavone bioavailability is presented in Figure 1.4 and the features in this diagram will be described in the next few sections.

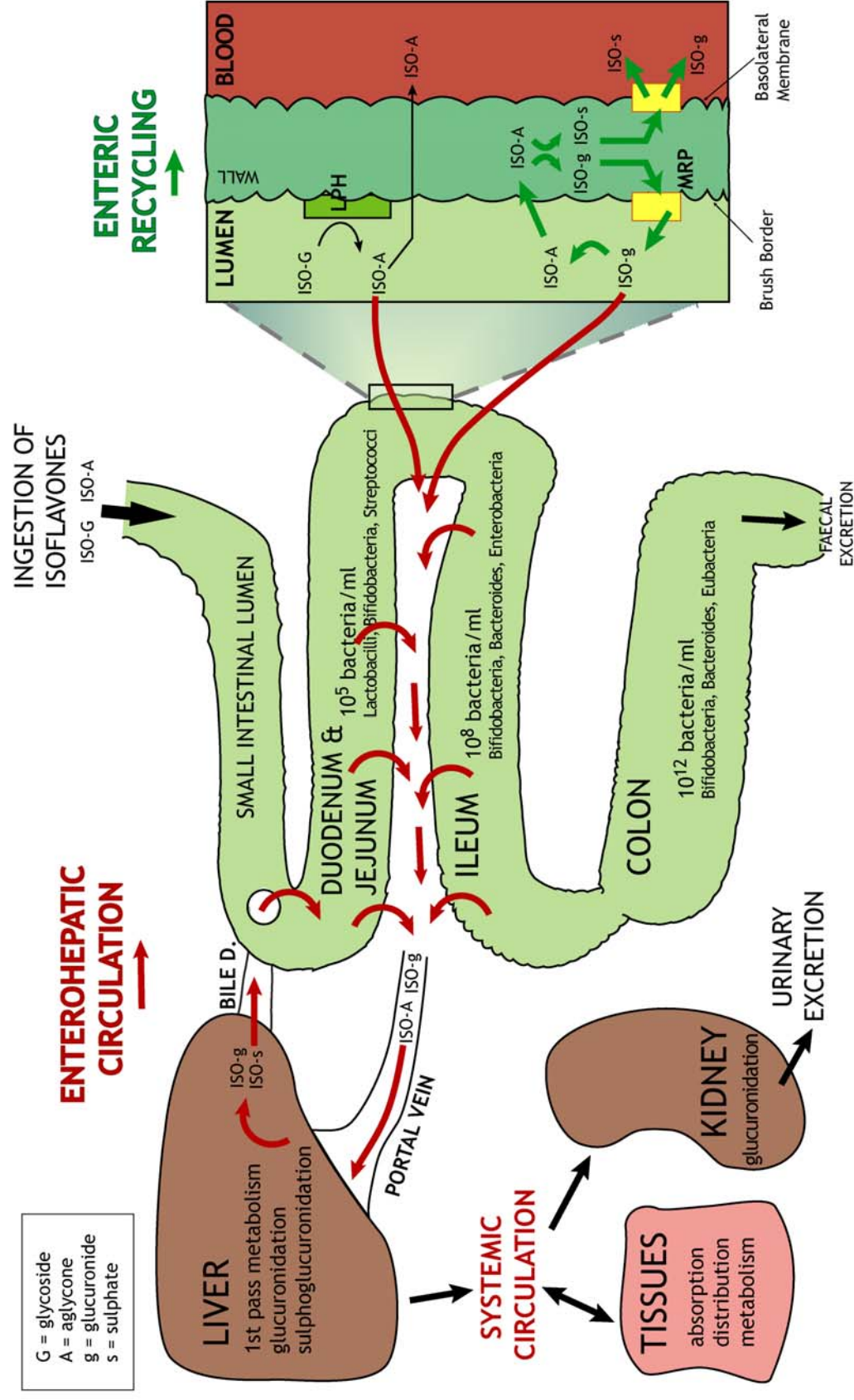


Figure 1.4 Diagrammatic Representation of The Physiological Processes Involved in Isoflavone Bioavailability.

MRP = multi-drug resistance protein LPH = lactase-phlorizin hydrolase

1.6.2 Isoflavone absorption

After soy intake, the isoflavone glucosides are poorly absorbed in the small intestine because of their hydrophilicity and large molecular weight (Liu and Hu, 2002; Xu *et al.*, 1995). Initial hydrolysis is thus necessary to release the free aglycones which are rapidly absorbed via passive diffusion across the intestinal brush border (Scalbert and Williamson, 2000) with high permeability (Liu and Hu, 2002). Glycosidase activity can occur in the food itself (of endogenous origin or added during processing), in the cells of the gastrointestinal mucosa or the enzyme can be secreted by the colon microflora (Scalbert and Williamson, 2000). Isoflavones have been measured in plasma as soon as 30 minutes after soy intake (King and Bursill, 1998) and others have shown an initial peak 1 hour post-meal (Franke *et al.*, 1999; Richelle *et al.*, 2002). This early increase may be due to the presence of a small percentage of aglycones in the soy meal (King and Bursill, 1998), but also suggests that hydrolysis and initial absorption occur readily in the duodenum and proximal jejunum (Rowland *et al.*, 2003; Setchell *et al.*, 2001; Watanabe *et al.*, 1998).

A number of mammalian β -glucosidases have been identified in the small intestine, including a broad specificity cytosolic β -glucosidase enzyme and the membrane-bound lactase phlorizin hydrolase (LPH) enzyme (Day *et al.*, 1998). LPH is present on the luminal side of the brush border in the small intestine and can deglycosylate genistein-7-glucoside and daidzein-7-glucoside within the gut lumen to release the more hydrophobic aglycones which can then diffuse into the epithelial cells (Day *et al.*, 2000). This activity suggests that the intestinal mucosa plays an important role in the deglycosylation of isoflavones (Day *et al.*, 1998; Scalbert and Williamson, 2000) and confirms the view that the isoflavone absorption begins in the proximal small intestine (Xu *et al.*, 1995) and occurs along its length (Setchell *et al.*, 2003b). The cytosolic β -glucosidase enzyme has been identified in the small intestine, liver and kidney of mammals (Day *et al.*, 1998), with the small intestine having a faster rate of hydrolysis of the 7-glucosides of daidzein and genistein than the liver (Day *et al.*, 2000). However, intestinal hydrolysis of

isoflavones would require initial uptake of the glucoside form, and although this has been demonstrated for other flavonoids (Paganga and Rice-Evans, 1997), it has not been conclusively shown for isoflavones (Setchell *et al.*, 2002b). Isoflavone glucosides that are not absorbed in the small intestine will pass through to the colon where bacterial β -glucosidases hydrolyse them, removing the sugar moiety for energy requirements (Parodi, 1999).

Plasma genistein is consistently reported as being higher than that of daidzein after soy intake (King and Bursill, 1998). This may simply reflect the generally higher levels of genistein and its conjugates compared with daidzein and its conjugates in most soy foods (Setchell *et al.*, 2001), but has also been reported when the intakes of daidzein and genistein are equivalent (Setchell *et al.*, 2003b). Experiments with pure compounds and stable isotopically-labelled isoflavones suggest that genistein is more bioavailable, with greater systemic exposure than daidzein (Setchell *et al.*, 2001; Setchell *et al.*, 2003b). A higher clearance rate of daidzein and its high volume of distribution contribute to the consistently lower serum concentrations compared with genistein (Setchell *et al.*, 2003a). In addition, LPH has shown a higher catalytic efficiency in hydrolysis of genistein than of daidzein (Day *et al.*, 2000), a factor that may also contribute to greater absorption of genistein. However, Xu, Wang and others (2000) reported similar plasma concentrations of daidzein and genistein after intake of soy milk powder that contained more genistein than daidzein.

1.6.3 Isoflavone metabolism

After initial absorption, the isoflavones undergo extensive first-pass metabolism, which accounts for their low bioavailability (Chen *et al.*, 2003). During phase II biotransformation, the hydroxyl groups of the isoflavones provide sites for glucuronidation and sulfation by glucuronosyl-transferases and sulphotransferases in the liver (Xu *et al.*, 1994) and/or intestine (Setchell *et al.*, 2001). Like most steroid hormones, the isoflavones undergo classical enterohepatic circulation and are conjugated

in the liver (Winter and Bokkenheuser, 1987). The glucuronide and sulphate conjugates can be transported via the systemic circulation to tissues, from where they will eventually be excreted via the kidneys, or they can be secreted in bile and returned to the intestine (Xu *et al.*, 1995). After deconjugation by intestinal bacteria, isoflavone aglycones can be reabsorbed, then returned to the liver via the portal vein for re-conjugation and either further enterohepatic circulation or renal excretion (Winter and Bokkenheuser, 1987).

However, recent evidence suggests that the intestine and subsequent enteric recycling may play a more significant role in isoflavone metabolism and bioavailability than previously realised (Chen *et al.*, 2003; Liu and Hu, 2002). In rats, the portal vein contains predominantly 7-O-glucuronide isoflavones (Barnes *et al.*, 1996), suggesting the primary site of glucuronidation is the intestinal wall (Coldham and Sauer, 2000). This was confirmed by evidence that in the rat small intestine, MRP (multi-drug resistance-related protein) conjugated and efficiently secreted glucuronidated isoflavones into the intestinal lumen (Liu and Hu, 2002). Chen and co-authors (2003) reported that *in vitro*, significant amounts of genistein were glucuronidated and sulphated by intestinal cells and then excreted into both the apical and basolateral sides of the enterocyte, and suggested that the upper intestinal metabolism could surpass that of the liver. This intestinal conjugation of isoflavones, their secretion back into the intestinal lumen and further reabsorption and re-conjugation constitutes enteric recycling (Chen *et al.*, 2003; Liu and Hu, 2002), which, in combination with enterohepatic recycling, prolongs the systemic exposure to isoflavones (Turner *et al.*, 2003).

In circulation, the glucuronides are the predominant metabolites of isoflavones (Spencer *et al.*, 1999; Zhang *et al.*, 2003), followed by the sulphated conjugates (Adlercreutz *et al.*, 1993). Other conjugates also exist, including sulphoglucuronides (Adlercreutz *et al.*, 1993), while the aglycones only represent a small proportion of total plasma isoflavones (Shelnutt *et al.*, 2002) and there is a high percentage of binding of isoflavones to plasma proteins (Coldham and Sauer, 2000). After soy intake, the percentage of total plasma daidzein as glucuronides (Zhang *et al.*, 2003) and sulphates (Shelnutt *et al.*, 2002) is greater than that of genistein. For estrogens, the sulphate conjugates are excreted slowly

compared with the glucuronide conjugates, and can serve as a source of biologically active estrogens when hydrolysed in target tissues (Adlercreutz *et al.*, 1987). However, the sulphate conjugates of both daidzein and genistein are cleared faster than the glucuronides (Shelnutt *et al.*, 2002). In addition, while the concentration of daidzein sulphate in plasma is much higher than genistein sulphate, it is cleared faster, but these two compounds show similar urinary recovery (Shelnutt *et al.*, 2002); this may suggest greater tissue distribution of daidzein sulphate.

Maximal plasma concentrations of daidzein and genistein are generally reached between 6 and 8 hours after soy intake in humans (King and Bursill, 1998; Setchell and Cassidy, 1999; Setchell *et al.*, 2003a; Setchell *et al.*, 2003b; Xu *et al.*, 1994; Xu *et al.*, 1995); daidzein often reaches its peak concentration later but has a faster plasma disappearance rate than genistein (Shelnutt *et al.*, 2002). The half-lives of plasma elimination are dependent on the conjugate; these are 3 - 9 hours for daidzein and 8 - 11 hours for genistein after intake of soy foods or pure isoflavone glycosides (Setchell *et al.*, 2003b; Shelnutt *et al.*, 2002; Watanabe *et al.*, 1998), but 9 and 7 hours for pure daidzein and genistein aglycone administration respectively (Setchell *et al.*, 2001).

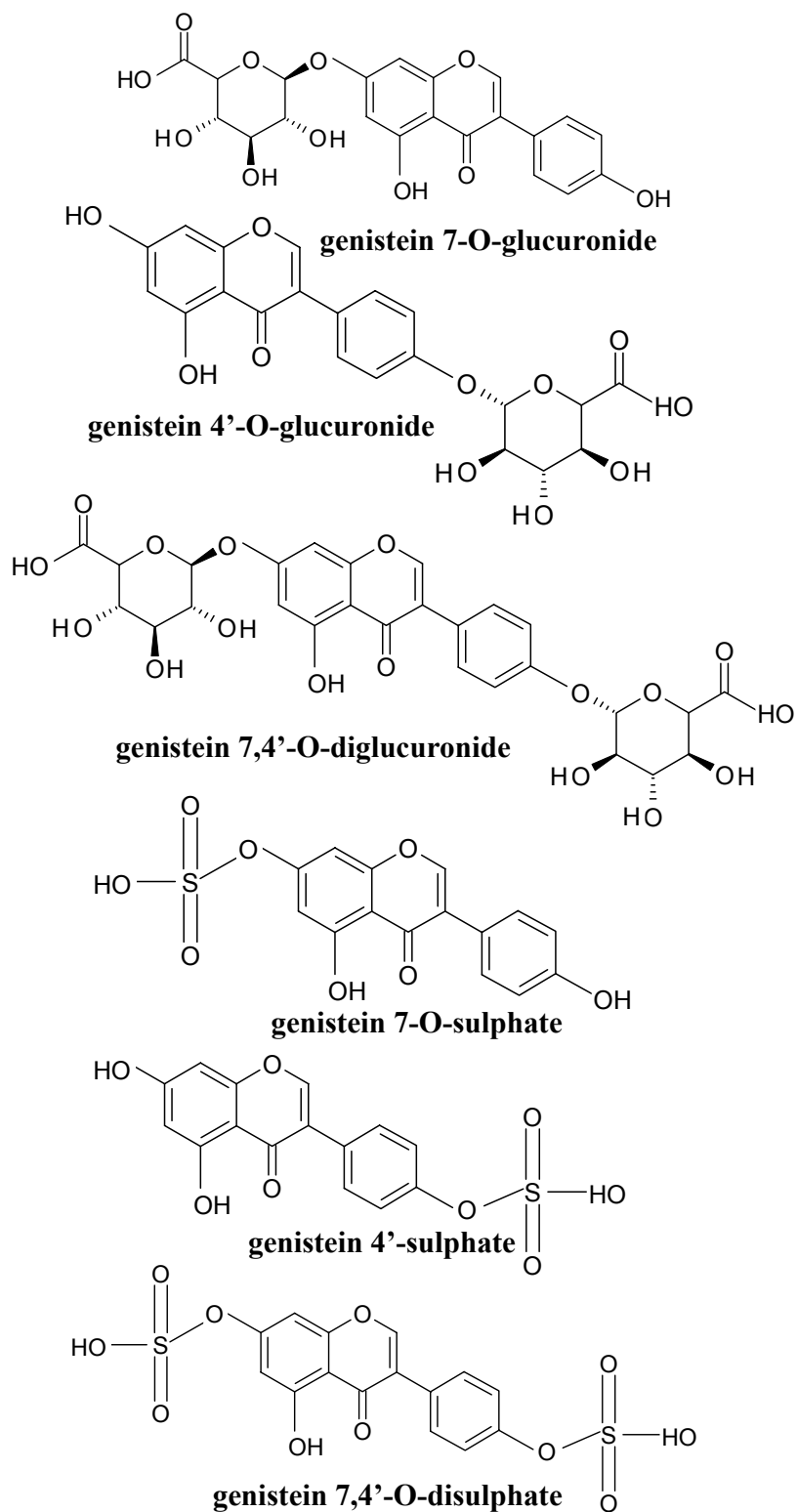


Figure 1.5 Glucuronide and sulphate conjugates of genistein. Analogous structures of daidzein are also present in the circulation.

Whether returned to the intestinal lumen via excretion in bile or by enterocytes, the isoflavone conjugates are deconjugated by enzymes in the intestinal wall (β -glucuronidases) or bacterial enzymes (β -glucuronidases and sulphatases) (Chen *et al.*, 2003; Winter and Bokkenheuser, 1987). Aglycones that are not reabsorbed will reach the colon, along with any conjugates from liver or intestinal biotransformation that are not deconjugated (Liu and Hu, 2002) and the fraction of isoflavone that is neither hydrolysed nor absorbed in the small intestine initially (Decroos *et al.*, 2005). Between 30 and 50% of estrogen metabolites are excreted in bile and about 80% of biliary conjugates are reabsorbed (Adlercreutz *et al.*, 1987) with less than 10% excreted in the faeces (Thompson, 1994). Similarly for isoflavones, measurement of intact isoflavones has accounted for only between 15 and 30% of the ingested dose and faecal excretion is also low (Xu *et al.*, 1995). Thus, the majority of unaccounted isoflavone dose must be metabolised in the intestine (Rowland *et al.*, 2003) and/or more extensive metabolism must take place in the tissues, liver and circulation (Spencer *et al.*, 1999).

In the colon, secondary metabolites are more easily absorbed (Chen *et al.*, 2003) and colonic microflora further degrade the isoflavones to simpler compounds which may involve splitting of the heterocyclic oxygen containing ring (Hollman, 2001; Scalbert and Williamson, 2000). Both daidzein and genistein can be further metabolised to secondary metabolites via the intermediates dihydrodaidzein and dihydrogenistein, respectively (Figure 1.1). Daidzein can be metabolised by reduction to equol and ring cleavage to o-desmethylangolensin (ODMA) and genistein to p-ethyl phenol and 6'-hydroxy-o-desmethylangolensin (6'ODMA), but of these secondary metabolites, only equol is biologically active (Hutchins *et al.*, 1995; Setchell *et al.*, 2002a). Metabolism to equol and ODMA may be inversely related, which suggests two alternative pathways in daidzein metabolism (Kelly *et al.*, 1995). In addition, a number of other minor metabolites of both daidzein and genistein have been identified in plasma, urine and faeces (Chang and Nair, 1995; Heinonen *et al.*, 1999; Joannou *et al.*, 1995; Kelly *et al.*, 1993). The higher molecular weight and lower water solubility of genistein may promote excretion of genistein conjugates in bile and provide more opportunity for bacterial degradation (Xu *et al.*, 1994), whereas daidzein appears to be less subjected to bacterial

metabolism *in vivo*. Thus it is more likely to be absorbed and therefore potentially more bioavailable (Decroos *et al.*, 2005). However, it has also been suggested that the carbonyl moiety of genistein is protected by hydrogen bonding with the adjacent hydroxyl group (Figure 1.1), thus rendering it less reactive in contrast to the carbonyl moiety of daidzein that may be readily metabolised by reduction and dehydration to equol (Coldham *et al.*, 2002).

1.6.4 Isoflavone distribution

Isoflavones have been quantified in plasma, urine, bile, faeces and prostatic fluid (Morton *et al.*, 1997; Watanabe *et al.*, 1998) as well as in breast tissue and breast milk (Franke and Custer, 1996; Franke *et al.*, 1998; Maubach *et al.*, 2003; Pumford *et al.*, 2002). They have also been demonstrated to cross the blood brain barrier and placenta (Adlercreutz *et al.*, 1999; Setchell and Cassidy, 1999). In men from soy-consuming countries, levels of isoflavones are higher in prostatic fluid than those of Western populations and concentrated approximately 2-fold relative to plasma (Morton *et al.*, 1997). Setchell and co-authors (2001) have estimated a large volume of distribution of the isoflavone aglycones and secondary metabolites indicating they have the potential to modulate actions at a range of tissues. Busby and colleagues (2004) determined that the volume of distribution of free plasma daidzein and genistein was nearly twice that of their respective conjugates. The aglycones are also cleared from the plasma much more rapidly than conjugated isoflavones, suggesting that free isoflavones enter and perhaps are sequestered in tissues.

1.6.5 Elimination and recovery of isoflavones

Isoflavones are excreted in urine almost exclusively as acidic conjugates, mainly glucuronides, with lesser amounts of sulphates and sulphoglucuronides (Adlercreutz *et al.*, 1993). After soy intake, urinary excretion of daidzein and genistein is typically

highest 7 - 8 hours post-meal (Lu *et al.*, 1995; Watanabe *et al.*, 1998). King and Bursill (1998) reported that mean excretion rates for genistein and daidzein increased progressively reaching a peak 6 – 12 hours after the meal and Watanabe and co-authors (1998) found that a plateau was reached 8 - 12 hours after intake. The majority of the urinary excretion of daidzein and genistein occurs within the first 24 hours after soy ingestion (Lu *et al.*, 1995; Setchell *et al.*, 2003b). Although King and Bursill (1998) suggested a constant elimination rate between 11 and 35 hours after a meal, Watanabe and colleagues (1998) showed that during 48 hours post soy intake, subjects characteristically showed two or three peaks of daidzein and genistein excretion, and attributed this to enterohepatic circulation.

In contrast to the higher plasma concentrations of genistein compared with daidzein, most studies report greater urinary excretion of daidzein than genistein (Franke *et al.*, 1999; Xu *et al.*, 1994). In addition, a higher proportion of daidzein occurs in urine in the unconjugated form (Adlercreutz *et al.*, 1993). It has been suggested that the lower molecular weight (254 vs. 270) and greater water solubility of daidzein could account for its higher urinary excretion (Xu *et al.*, 1994), while the lower hydrophilicity of genistein may promote its excretion in bile (King and Bursill, 1998). Isoflavones are also excreted faecally (Watanabe *et al.*, 1998), predominantly in the unconjugated form, less than 10% being conjugated (Adlercreutz *et al.*, 1995).

The urinary recovery of isoflavones, as the proportion of the total amount excreted relative to the amount ingested, is quite low and has been reported to be between 10 and 50% (Cassidy *et al.*, 2000; Hendrich *et al.*, 1998), which may indicate significant colonic bacterial degradation, and possible metabolism to other unidentified compounds (Lampe *et al.*, 1998; Xu *et al.*, 1994). Hendrich and co-authors (1998) suggested that biliary excretion is likely to be the main limiting factor with respect to the percentage of isoflavones that are systemically available after intake. In addition, cytochrome p450 enzymes appear to play an important role in the oxidative metabolism of the soy isoflavones daidzein and genistein and might explain their low recoveries (Kulling *et al.*,

2000). Total faecal excretion of isoflavones is typically less than 5% (Xu *et al.*, 1994; Xu *et al.*, 1995).

1.6.6 Production of equol from daidzein

The conversion of daidzein to equol may be important as equol has significantly enhanced antioxidant activity compared with daidzein (Arora *et al.*, 1998; Mitchell *et al.*, 1998). Equol has equivalent antioxidant potency as estradiol and quercetin (Vedavanam *et al.*, 1999; Wiseman and O'Reilly, 1997) and is approximately 100-fold more estrogenic than daidzein on binding to the ER (Sathyamoorthy and Wang, 1997). A case-control study found a substantial reduction in breast cancer risk among women with high excretion of isoflavones, with those excreting equol having the greatest reduction (Ingram *et al.*, 1997). Similarly, Akaza and co-authors (2002) reported that the percentage of equol-producers in a case-control study was significantly lower among patients with prostate cancer compared with controls. In a soy supplementation study, the greatest increases in the length of the follicular phase of the menstrual cycle, a change associated with lower breast cancer risk, were reported in two subjects who also had the highest urinary equol excretion (Cassidy *et al.*, 1994).

Equol is exclusively produced by intestinal bacteria (Decroos *et al.*, 2005), but not all people can metabolise daidzein to equol. It is consistently reported that between 30 and 40% of individuals excrete equol after consuming soy products (Lampe *et al.*, 1998; Lampe *et al.*, 2001; Slavin *et al.*, 1998). The distinct presence or absence of equol has allowed researchers to classify subjects according to their equol-producing ability, allowing for separate analysis of endpoints between these two groups (Karr *et al.*, 1997; Kelly *et al.*, 1995; Lampe *et al.*, 1998; Rowland, 1999; Setchell *et al.*, 2002a).

Equol is unique in having a chiral centre due to the absence of a double bond in the heterocyclic ring, and therefore two distinct optically active isomers occur. The naturally occurring enantiomer from endogenous conversion of daidzein is S(-)equol (Muthyala *et*

al., 2004). The R and S isomers differ conformationally, with the latter being more non-planar (Setchell *et al.*, 2002a), suggesting a higher affinity of this isomer for estrogen receptors (Barnes and Peterson, 1995). It has recently been confirmed that S(-)equol has a high binding affinity and strong preference for ER- β , whereas R(+)-equol binds more weakly and with a preference for ER- α (Muthyala *et al.*, 2004). This non-planar property of S(-)equol also confers it with greater flexibility for conformational changes, which could enable it to penetrate into the interior of cell membranes with greater ease than other isoflavones more rigid in structure (Arora *et al.*, 1998).

The pharmacokinetics of equol are similar to the other isoflavones; however, equol has a slower plasma clearance (Lampe *et al.*, 2001; Setchell *et al.*, 2002a) and a longer half-life (Kelly *et al.*, 1995) than daidzein, with maximal plasma levels generally reached between 24 hours and 3 days post-intake (Kelly *et al.*, 1995; Setchell *et al.*, 2001). Metabolism of daidzein to equol is time-dependent (Setchell *et al.*, 2003a) with a lag time in its appearance of at least 6 - 8 hours after intake of a bolus dose, consistent with its colonic origin (Setchell *et al.*, 2001; Setchell *et al.*, 2003b). Equol is readily absorbed from the gastrointestinal tract, more efficiently through the colon wall compared with daidzein (Decroos *et al.*, 2005), and conjugated to glucuronic acid in the liver (Axelson *et al.*, 1982). Hepatic metabolism may be more important for compounds like equol, that are mainly absorbed from the large intestine (Chen *et al.*, 2003) and this may contribute to the longer pharmacokinetics of equol. The formation of equol may be dependent on initial levels of daidzein (Tsangalis *et al.*, 2002) and equol bioavailability is reportedly greater after ingestion of daidzein glucoside rather than the aglycone, possibly due to the longer transit time of the former (Zubik and Meydani, 2003). Once formed, equol appears to be metabolically stable, undergoing no further biotransformation, other than phase II metabolism (Setchell *et al.*, 2002a); however, it has been recently suggested that equol is further metabolised, possibly in the liver, leading to catecholic structures of either ring A or B (Adlercreutz *et al.*, 2004).

Equol also remains elevated in the urine for a longer duration after a soy challenge compared with daidzein and genistein (Lampe *et al.*, 2001), with maximum urinary

excretion having been reported between 24 and 72 hours or more after intake (Axelson *et al.*, 1982; Kelly *et al.*, 1993; Kelly *et al.*, 1995; Xu *et al.*, 1994). It is excreted in urine almost exclusively as the monoglucuronide conjugate (Axelson *et al.*, 1982). Watanabe and co-authors (1998) calculated the percent metabolic conversions of daidzein to ODMA and equol as 4 and 7%, respectively and reported the faecal excretion of equol was much higher 5 – 6 days compared with 4 days after intake, suggesting that much of the faecal equol may represent biliary excretion.

1.6.7 Role of the gut microflora in isoflavone bioavailability

The essential role of the gut microflora in isoflavone absorption and metabolism has been demonstrated through the use of antibiotics which dramatically decrease plasma isoflavone concentrations and urinary excretion of the bacterial metabolites (Adlercreutz, 1998; Rowland, 1999; Winter and Bokkenheuser, 1987). Additionally, studies using “germ-free” rats have shown an absence of isoflavone absorption, which is revived when these rats are inoculated with human gut flora (Bowey *et al.*, 2003). The enzymes most pertinent to isoflavone bioavailability are the β -glucosidases, necessary for glucoside hydrolysis and aglycone absorption, and β -glucuronidases and sulphatases, required for reabsorption of the hepatic conjugates and biliary excretion (Xu *et al.*, 2000). The initial hydrolysis to the aglycone by β -glucosidases appears to be the rate-limiting step in isoflavone absorption (Izumi *et al.*, 2000; Setchell *et al.*, 2001; Steer *et al.*, 2003). β -glucosidase enzymes are produced by several groups of bacteria including bacteroides, bifidobacteria and lactobacilli (Steer *et al.*, 2003; Xu *et al.*, 1995), of which bacteroides and bifidobacteria comprise the majority of microorganisms in the human gastrointestinal tract (Friend and Chang, 1984).

The extent to which compounds are metabolised by gut bacteria depends on, amongst other factors, the region of the gut from which the compound is absorbed, the distribution and type of bacteria and the availability of the necessary enzyme (Hawksworth *et al.*, 1971). The microflora of the large intestine is acquired after birth, with a pattern

resembling adult flora established after weaning (Isolauri, 2001; Salminen *et al.*, 1998). The species composition that develops is largely controlled by diet (Salminen *et al.*, 1998), which especially affects gut microfloral enzyme activities during the transition to a more diversified diet, between 6 and 12 months (Mykkanen *et al.*, 1997). Bacterial numbers and composition vary considerably along the human gastrointestinal tract, with numbers increasing along the length of the small intestine to approximately 10^8 per mL of contents at the ileocecal region (Salminen *et al.*, 1998). The large intestine usually contains more than 400 species of bacteria (Parodi, 1999), typically numbers of approximately 10^{12} per gram contents (Gibson, 1998; Salminen *et al.*, 1998). In the proximal small intestine (duodenum and jejunum) where absorption is at its peak, the microflora is dominated by species of *Streptococcus*, *Lactobacillus* and *Bifidobacterium*, while in the distal small intestine (ileum) and colon, *Bacteroides* and *Bifidobacterium* species dominate (Parodi, 1999; Turner *et al.*, 2003). Of the gut microflora, enterococci (found in high levels in the colon) have the highest β -glucosidase activity, followed by lactobacilli, bacteroides and bifidobacteria. *In vitro*, bifidobacteria can metabolise the isoflavone glycosides and further metabolism of daidzein to equol is correlated with its β -glucosidase activity (Tsangalis *et al.*, 2002).

Recently, much research has been directed towards the elucidation of the bacteria responsible for the production of equol from daidzein. Strains of bifidobacteria (Tsangalis *et al.*, 2002), streptococci, ruminococci, bacteroides (Ueno and Uchiyama, 2001), enterococci and lactobacilli (Decroos *et al.*, 2005) as well as *Escherichia coli* (Hur *et al.*, 2000), have all been identified as having this capability. However, it appears that more than one bacterial species is involved in the metabolism of daidzein to equol (Decroos *et al.*, 2005; Hur *et al.*, 2000) and thus the presence or absence of equol will depend on an individual's microflora composition and bacterial enzyme expression (Turner *et al.*, 2003). Urinary excretion of equol seems to be inversely related to that of daidzein and ODMA (Kelly *et al.*, 1993; Kelly *et al.*, 1995; Lampe *et al.*, 2001; Slavin *et al.*, 1998), suggesting that daidzein is preferentially metabolised to either ODMA or equol, depending on gut microflora and/or inherent characteristics. Decroos and co-authors (2005) reported that equol and ODMA are indeed formed by different bacteria,

but that these species can co-exist. Thus, equol-excretor status may be a marker of a particular colonic microfloral profile (Lampe *et al.*, 2001) although it is not known if this can be modulated by diet.

1.7 FACTORS AFFECTING ISOFLAVONE BIOAVAILABILITY

A number of factors can influence the absorption of food components, including dietary habits, the food matrix, intestinal fermentation and transit time (Zubik and Meydani, 2003). In isoflavone bioavailability studies, the soy food used and its isoflavone composition may be important determinants of the resulting isoflavone pharmacokinetics and physiological effects. The influence of diet is important due to interactions between dietary components and also because of the impact of diet on gut microflora, which in turn plays a crucial role in isoflavone bioavailability. Inherent and genetic characteristics which determine pathways of absorption and metabolism will also contribute to variability in isoflavone bioavailability. A better understanding of these differences will assist in the interpretation of outcomes of dietary studies.

1.7.1 Composition of soy foods and variability in isoflavone concentrations

The relative proportions of different isoflavone conjugates in soy may have an effect on resulting bioavailability. It is not known what effects 6"-O-substitution has on the susceptibility of the isoflavone conjugates to intestinal hydrolysis and absorption; however, differences in bioavailability and metabolism dependent on the nature of their chemical form would be anticipated (Barnes *et al.*, 1994). There are differences in the location of absorption of these conjugates, as the aglycones are absorbed readily from the upper small intestine, the β -glucoside conjugates from the distal small intestine after hydrolysis to the aglycone, and the malonylglucoside and acetylglucoside conjugates from the large intestine after hydrolysis (Coward *et al.*, 1998). This could affect the subsequent bioavailability of the aglycones as absorption efficiency and the distribution

of conjugating enzymes differs between different areas of the gastrointestinal tract (Liu and Hu, 2002). More specifically, the proportions of daidzein, genistein and glycitein, will also greatly affect the resulting isoflavone bioavailability and overall physiological effects, due to their different chemical structures and *in vivo* properties.

Most soy-containing foods consumed in Western diets are made from soy protein which contains glycosidic isoflavones, as opposed to fermented soy products in which aglycones predominate (Setchell *et al.*, 2001). The systemic bioavailability of isoflavones is not the same after consumption of β -glucosides compared with aglycones, as the former have longer kinetics of absorption and excretion (Setchell *et al.*, 2001; Steer *et al.*, 2003), which may prolong endogenous exposure and increase bioavailability. The aglycones are absorbed faster and with greater maximum concentration; however, plasma levels do not remain elevated for as long (Izumi *et al.*, 2000). In addition, the conjugation may also determine the extent to which the isoflavones are metabolised to secondary metabolites and Zubik and Meydani (2003) suggested that the longer transit time for glucosides may provide more opportunity for bacterial metabolism. The food matrix will also influence subsequent bioavailability, with isoflavones from supplements likely to be absorbed at a faster rate than those ingested in a food matrix (Richelle *et al.*, 2002).

1.7.2 Level and duration of isoflavone intake

In a dose-dependent analysis using pure isoflavone supplements, Setchell and co-authors (2003a) reported no effect of isoflavone dose on the time to maximum plasma concentration, but there was a decreased fractional absorption when the dose was doubled, indicating that a plateau was reached. Similarly, intake of soy nuts at three different amounts resulted in similar half-lives, clearance and volume of distribution, independent of dose (Setchell *et al.*, 2003b). Although Hendrich and co-authors (1998) reported that human bioavailability of isoflavones is linearly related to dose within a broad range, it is possible that non dose-dependent plasma kinetics at higher intakes may

result in saturable plasma levels (Setchell and Cassidy, 1999; Setchell *et al.*, 2001). However, once absorbed, dietary polyphenols including isoflavones are not expected to saturate metabolic pathways, but dose will determine the primary site of metabolism (Scalbert and Williamson, 2000). Large doses will be metabolised primarily in the liver, while smaller doses may be metabolised predominantly by intestinal mucosa with the liver playing a secondary role (Scalbert and Williamson, 2000). This may be important in relation to the metabolites produced; while oxidative metabolism predominates in the mammalian liver, gut microflora is active in reductive reactions (Rowland, 1986), such as the metabolism of daidzein to equol. It is not known how subsequent bioavailability and tissue distribution are affected by the location of isoflavone metabolism.

Long-term soy intake may also affect isoflavone bioavailability if enzymes important in isoflavone bioavailability are induced by prior exposure. β -glucuronidase has been demonstrated to be inducible by glucuronide conjugates (Silvi *et al.*, 1999) and this may be similar for other enzymes. Four weeks of soy milk ingestion increased the absorption half-lives of daidzein and equol as well as the proportion of free unconjugated isoflavones (Lu *et al.*, 1995), which may result in greater isoflavone bioavailability. Hendrich and co-authors (1998) also reported that more frequent doses prolong plasma clearance time. However, there does not appear to be an effect of long-term intake (up to 10 weeks) of soy foods on absolute plasma concentrations (Wiseman *et al.*, 2004).

1.7.3 Inherent differences in gut microfloral metabolic activity

The species composition of gut microflora and total activity of enzymes vary widely between individuals (Day *et al.*, 1998; Salminen *et al.*, 1998) and genetic polymorphism has been demonstrated for a number of host enzymes (Scalbert and Williamson, 2000). The expression of β -glucuronidases in human cells is often regulated during development (Scalbert and Williamson, 2000). LPH, which has been implicated as having a major role in intestinal absorption of isoflavone aglycones, is also primarily responsible for hydrolysis of lactose, and deficiency of this enzyme causes lactose intolerance (Day *et*

al., 1998). This condition affects approximately 5% of Europeans and 90% of Africans and Asians in adulthood (Scalbert and Williamson, 2000). For those individuals who are deficient in LPH, isoflavone absorption in the small intestine may be reduced which would result in more isoflavones reaching the colon for microbial metabolism (Day *et al.*, 2000), including the conversion of daidzein to equol. Furthermore, Thadepalli and co-authors (1979) reported differences between the gastrointestinal microflora of North Americans and Western Europeans compared with South Indian and Guatemalan individuals. Thus, genetic differences, apparent in gut microflora and enzyme activity may also influence isoflavone bioavailability.

1.7.4 Effects of diet and gut microflora

Human intestinal microflora composition is relatively stable (Lampe *et al.*, 1998) and differences in normal dietary patterns do not appear to influence the composition of the intestinal microflora extensively. However, diet and antibiotics can substantially modify the metabolic activity of bacteria including that of β -glucosidases and β -glucuronidases (Parodi, 1999; Wang *et al.*, 2002) and enterohepatic circulation (Goldin *et al.*, 1982; Gorbach and Goldin, 1992). For instance, vegans have lower faecal β -glucuronidase activity (Parodi, 1999) and transition to a vegan diet in adults alters faecal bacterial enzyme activities (Mykkanen *et al.*, 1997). Dietary induced changes in gut microflora and enterohepatic circulation may subsequently affect isoflavone bioavailability. In addition, dietary substrates such as fibre can also modulate intestinal and faecal characteristics including transit time, bulk and water content (Parodi, 1999) and conversely, these faecal characteristics can influence substrate availability, redox potential in the colon (Gibson, 1998) and accessibility of bacteria for dietary substrates (Parodi, 1999).

The principal substrates for colonic bacterial growth are dietary carbohydrates that have not been digested in the upper intestinal tract (Gibson, 1998; Yue and Waring, 1995). Most genera of the large intestinal microflora are saccharolytic and obtain energy by

fermentation of such dietary carbohydrates, including non-starch polysaccharides and resistant starch (Parodi, 1999). Recently, much research has been focused on modulation of gut microflora from either oral intake of probiotic bacteria or ingestion of a prebiotic, which can induce the activities of specific endogenous probiotic gut microflora, with expected subsequent effects on isoflavone bioavailability. However, two recent studies showed no change in plasma or urinary isoflavones when a soy diet was supplemented with probiotic bacteria intake (Bonorden *et al.*, 2004; Nettleton *et al.*, 2004). This will be discussed in more detail in Chapters 3 and 5. Prebiotic effects in the gastrointestinal tract may be particularly relevant to equol production as the metabolism of daidzein to equol relies exclusively on gut microflora (Decroos *et al.*, 2005).

The determinants of equol production are still largely unknown. It has been suggested that genetics play an important role (Kelly *et al.*, 1993), which suggests that the ability to metabolise daidzein to equol is an inherent characteristic. However, if gut microflora is a determining factor, it may be possible to alter bacterial composition via dietary intervention in a manner favourable to equol production. An individual's ability to produce equol has been associated with less dietary fat intake and with a greater intake of carbohydrate (Lampe *et al.*, 2001; Rowland, 1999), non-starch polysaccharides (Rowland, 1999) and dietary fibre (Lampe *et al.*, 1998). In particular, the type and amount of carbohydrate available to intestinal microflora may be important for equol-producing capacity (Lampe *et al.*, 2001; Rowland, 1999) and experiments using an *in vitro* colonic fermentation system found that a high carbohydrate environment increased fermentation and the rate of conversion of daidzein to equol (Setchell and Cassidy, 1999). Thus, increased intake of carbohydrate, either acutely or via habitual diet, may also increase equol production *in vivo*. *In vivo* effects on the metabolism of daidzein to equol could influence the potential health protective effects of soybean isoflavones if indeed equol has greater impacts on physiological function (Wiseman, 1999). However, attempts to increase equol production via dietary intervention with various soy-derived foods or the addition of wheat to soy diets have so far been unsuccessful (Lampe *et al.*, 1998; Lampe *et al.*, 2001; Slavin *et al.*, 1998; Tew *et al.*, 1996).

1.7.5 Direction of research

A vast amount of evidence suggests beneficial effects of soy consumption, particularly in relation to hormone dependent cancers and cardiovascular disease. However, there is much variation in the results of dietary interventions in terms of isoflavone bioavailability and clinical outcome measures. Background diet may be one of the most important factors affecting isoflavone bioavailability and thus, their physiological effects, and is an easily modified condition. In addition, the role of gut microflora may be particularly important in the production of the daidzein metabolite equol, which may confer more health benefits than its precursor.

1.8 THESIS HYPOTHESES AND AIMS

1.8.1 Thesis hypotheses

It is hypothesised here that soy isoflavone bioavailability will be dependent on the food matrix in which the soy component is contained and the relative proportions of daidzein and genistein. Further, it is also hypothesised that dietary intervention combining soy with a probiotic or prebiotic food component may enhance soy isoflavone bioavailability and the lipid-lowering effects of the isoflavone component of soy. Lastly, it is hypothesised that concurrent soy intake and either probiotic or prebiotic consumption or n-3 fatty acid supplementation will result in synergistic health beneficial effects on lipid profiles in hyperlipidemic subjects. Specific background information pertinent to the development of each hypothesis is presented in the appropriate chapter.

1.8.2 Thesis aims

This thesis aims to examine factors affecting soy isoflavone bioavailability. The effects of the soy matrix itself (soy milk and soy cereal, which differ in their levels of soy

protein and isoflavones) and the effects of various dietary components (probiotic bacteria, resistant starch and DHA-rich oil) on soy isoflavone bioavailability will be determined. In addition, there is an aim to ascertain whether different food combinations with soy and/or isoflavone bioavailability are determining factors in lipid changes with soy intervention. Four human studies were undertaken to establish the influence of dietary components on soy isoflavone bioavailability and effects on lipids. These results may help to explain the inter-individual variability in isoflavone bioavailability and any subsequent health benefits.

1. Acute and Chronic Effects of Resistant Starch Intake on Soy Isoflavone Bioavailability in Females – A Pilot Study (Chapter 3).

This pilot study aimed to determine the effects of acute (single meal) and chronic (4 weeks) resistant starch intake on isoflavone bioavailability and metabolism from a single soy meal in healthy, young, omnivorous women. Resistant starch is a prebiotic and thus produces bacterial change in the gut; its combination with soy has not previously been reported. It was hypothesised that resistant starch intake may thus affect isoflavone bioavailability. From multiple plasma and urine samples collected after each soy meal, this study allowed an initial evaluation of the prebiotic effects of resistant starch on isoflavone bioavailability and the establishment of optimal sampling procedures for use in further studies.

2. Effects of Soy vs. Dairy Products on Isoflavone Bioavailability and Lipids in Mildly Hyperlipidemic Subjects: A 10-week Crossover Study (Chapter 4).

As previous soy dietary interventions using soy protein and/or isoflavone supplements have yielded inconclusive results in relation to lipids effects, it was hypothesised that whole soybean supplementation may be more beneficial in this regard. In addition, isoflavone bioavailability following consumption of soy milk containing whole soybeans may differ from that after intake of more isolated and extracted soy components. A crossover design was used to assess the effects of soy food intake compared with dairy

products on isoflavone bioavailability and lipids in mildly hyperlipidemic men and postmenopausal women.

3. Effects of Probiotic and Prebiotic Foods on Soy Isoflavone Bioavailability (Chapter 5).

Gut microflora plays a significant role in isoflavone bioavailability and metabolism and can be modified by other dietary components, particularly probiotics and prebiotics. Thus, it was hypothesised that dietary intake of such foods may alter isoflavone bioavailability. In addition, as both probiotic yoghurt and prebiotic resistant starch can have positive lipid effects, it was also hypothesised that dietary combination with soy would result in more enhanced lipid benefits than intake of soy alone. This study was conducted with hyperlipidemic men and postmenopausal women and followed a crossover design. There were two cohorts (probiotic and prebiotic) to compare the effects of a combination of soy and either a probiotic yoghurt or resistant starch with soy alone on isoflavone bioavailability and lipid levels.

4. Combination of Soy Cereal and DHA-rich oil on Plasma and Urinary Isoflavones and Lipids in hyperlipidemic subjects – A Controlled, Crossover Study (Chapter 6).

The development of functional foods for health benefits is an area of current expansion and interest, particularly in relation to lipid-lowering formulations. Soy and n-3 fatty acids both have independent lipid-lowering effects, hence it was hypothesised that dietary intervention with both soy and the n-3 fatty acid DHA may be a novel combination for the delivery of health benefits in relation to cardiovascular disease. This crossover study determined the effects of a combination of soy cereal and DHA-rich oil on isoflavone bioavailability and lipids, compared with control cereal and oil supplementation in hyperlipidemic men and postmenopausal women. In addition, the intake of a soy cereal higher in daidzein than genistein was hypothesised to increase equol production.

1.8.3 Summary

Overall, the findings of this thesis will be relevant to the understanding of some of the factors affecting isoflavone bioavailability from dietary soy intake. This is timely considering the current interest in differences between the physiological activities of the isoflavones, the importance of equol production and the use of dietary components to modulate gut microflora and thus isoflavone bioavailability. In addition, the incorporation of lipid measures in these bioavailability studies may assist in explaining the large variation between individuals in the lipid effects of dietary soy intake. This may further aid in establishing recommendations for the use of soy intake in lipid management on a more individual basis.

CHAPTER 2

GENERAL METHODS FOR ISOFLAVONE ANALYSIS BY HPLC - ECD

2.1 INTRODUCTION

In order to quantify isoflavones in food and biological samples, they must first be extracted from these complex matrices. This is dependent on the conjugation of the isoflavones which differs between food and biological fluids (Adlercreutz *et al.*, 1991a). A lack of standards for the isoflavone conjugates and the difficulty in measuring a diverse range of compounds means that most investigators report the combined total of free + conjugated isoflavones after enzymatic or acidic hydrolysis. Many methods exist for the extraction of various phytoestrogens, including isoflavones, lignans and coumestans. Although optimal quantification of each class of phytoestrogens requires slightly different extraction procedures and thus they are often extracted separately (Adlercreutz *et al.*, 1993; Mazur *et al.*, 1996), their structural similarities enable simultaneous extraction from the same sample (Gamache and Acworth 1998). It is also necessary to isolate the phytoestrogens from estrogens which due to similar polarity and molecular size are quite inseparable by simple extraction procedures (Joannou *et al.*, 1995). One of the main methodological problems in extracting isoflavones from food or biological matrices is the difficulty in achieving complete hydrolysis of all the conjugates without significant loss.

The two most commonly employed techniques for the separation and quantification of phytoestrogens in food, plasma and urine are GC/MS (gas chromatography/mass spectrometry) and HPLC (high performance liquid chromatography), both with their advantages and drawbacks. For this project, HPLC was the instrumentation most readily available. Although HPLC analysis with ultraviolet (UV) detection has commonly been

used for phytoestrogen analyses (Franke *et al.*, 1995; Franke *et al.*, 1998), the concentrations of isoflavones in complex matrices such as blood, tissue and urine, are too low for this detection method (Coward *et al.*, 1996). Isoflavones are electroactive due to the presence of the phenolic groups (Setchell and Welsh, 1987) and can therefore be detected with electrochemical detection (ECD) which has increased sensitivity and specificity compared with UV detection (Franke *et al.*, 1994). Gamache and Acworth (1998) reported a method using an 8-channel coulometric array with a multiple ECDs placed in series after the analytical column and each channel maintained at a different potential. This allows for resolution of co-eluting solutes to be obtained based on small differences in their oxidation-reduction behaviours over the range of potentials (between 260 and 680 mV). In addition, a gradient mobile phase enabled quantification of a range of compounds including hydrophilic isoflavone glycosides, the more hydrophobic estrogens and isoflavones, lignans and coumestrol. HPLC with ECD was the method of analysis employed for the studies in this thesis.

Reliable measurements of plasma, urinary and food isoflavone levels are essential for interpretation of human isoflavone bioavailability studies. Different researchers employ slightly different extraction procedures and analytical techniques for the quantification of isoflavones, depending on resource availability and outcome measures. Assessment of established methods is imperative in adapting these to new research. The quantification of isoflavones in food samples or in human plasma and urine from bioavailability studies was not an established or routine analysis at the University of Wollongong prior to the research for this thesis being undertaken. However in a study carried out by the author for an Honours project (1999), a method for the simultaneous analysis of isoflavones and lignans was initiated. This was based on the method described by Gamache and Acworth (1998). The studies in this thesis are focused exclusively on isoflavones, but this methodology provided the starting point for the current work, although further method development was required. An advantage of this starting point was to ensure that any lignans or their metabolites did not co-elute or interfere in the isoflavone quantification and analysis. The final methods for analytical procedures for samples obtained from the four human studies that comprise this thesis are also reported here.

2.2 METHOD DEVELOPMENT

2.2.1 HPLC Conditions

As isoflavones and lignans have similar chemical structures, they can both elute under the same HPLC and ECD conditions of mobile phase and voltage respectively. Thus, it is advantageous to be able to chromatographically separate lignans and isoflavones before quantification so that the levels of isoflavones are not over-reported due to co-elution with lignans. This is particularly relevant because in background diets, lignans are generally reported at a greater level than isoflavones (Hutchins *et al.*, 1995) and the combination of soy and linseed is popular in many foods in Australia. Thus, it was the aim of this method development to enable simultaneous quantification of isoflavones (daidzein, genistein and equol) and lignans (enterodiol and enterolactone) from the same sample, even though lignans values were not measured in the four studies in this thesis.

The HPLC conditions described by Gamache and Acworth (1998) were adapted for use in the current system. Gamache and Acworth (1998) used a gradient mobile phase in which mobile phase A was 50 mM sodium acetate, pH 4.8 with acetic acid / methanol (80/20 v/v) and mobile phase B was 50 mM sodium acetate, pH 4.8 with acetic acid / methanol / acetonitrile (40/40/20 v/v). The gradient program went from 20% to 100% mobile phase B over 25 min. A variety of mobile phases were trialed based on those reported by Gamache and Acworth (1998) including those using a gradient program, but with the HPLC system that was used, an isocratic mobile phase resulted in optimal peak separation and quantification. The final isocratic mobile phase was Na-AC (50 mM, pH 4.8)/MeOH/ACN (42:50:08) and isoflavone elution times were daidzein, 11 min; equol, 13.5 min; genistein, 16.5 min; ODMA, 18 min. Samples from the first 2 studies (Chapter 3 and Chapter 4) were run under these mobile phase conditions with a Coulochem II electrochemical detector (ESA, Activon Australia). Samples from the last 2 studies were run using an Electrochemical Flow Cell (VT-03, INTRO, Antec Leyden). Details are provided in the final method section (2.3.3).

2.2.2 Extraction of isoflavones from plasma and urine

Enzymatic hydrolysis of isoflavones in plasma and urine samples, typically with glucuronidase and sulfatase enzymes from *Helix Pomatia* juice or glucosidases (Adlercreutz *et al.*, 1991a), is more convenient for large numbers of samples compared with acid hydrolysis. For enzymatic hydrolysis using *Helix Pomatia* juice, others have used between 200 and 1000 μL of plasma and between 1,000 and 5,000 units β -glucuronidase per mL of plasma for 16 h or overnight extractions (King and Bursill, 1998; Morton *et al.*, 1994) or 20,000 units for 3 h (Gamache and Acworth 1998). For optimization of enzyme extraction, different volumes of initial plasma aliquot with varying enzyme amounts and extraction durations were tested using *Helix Pomatia* enzyme, which provided 374,000 U/g β -glucuronidase (with minimum sulfatase activity of 10,000 U/g). Enzyme solutions were made up in 0.1 M sodium acetate buffer (pH 5.0) containing 0.1% (w/v) ascorbic acid and 0.01% (w/v) EDTA (Gamache and Acworth 1998) for addition of 200 μL to the plasma sample. Plasma samples from 4 different subjects were used; each sample had been previously analysed and contained moderate levels of isoflavones. Thus analysis was more qualitative than quantitative as samples were compared within subjects initially and then overall (Table 2.1). It was concluded that extraction of 400 μL plasma with 1,000 units of β -glucuronidase for 16 h resulted in the greatest extraction of the isoflavones daidzein and genistein from the sample.

Table 2.1 Extraction optimization for daidzein and genistein.

Plasma aliquot (μL)	Enzyme amount (units)	Extraction duration (h)	Plasma sample [†]				Signal to noise ratio	Peak area
200	20,000	3		B	C		+	-
200	10,000	8		B	C	D	-	-
200	1,000	16	A	B			+	++
200	400	16	A	B		D	++	++
400	400	16	A		C	D	+	+
400	1,000	16	A		C	D	+++	+++

[†]Plasma samples were from 4 different subjects (A,B,C,D); each sample had been previously analysed and contained moderate levels of isoflavones. +++ corresponds to best response.

2.2.3 Voltage Optimisation for Antec-Leydon Electrochemical Flow Cell

During the course of this project, changes were made to the HPLC system to allow for greater ease in its use and to resolve problems and improve chromatography. Stainless steel tubing was replaced with PEEK tubing which reduced the volume through which the sample flowed and affected elution times. In addition, the conditioning and analytical cells incorporating the Coulochem II ECD were replaced with a single electrochemical flow cell (VT-03, Antec Leyden, Zoeterwoude, The Netherlands) which dramatically reduced pressure and background noise. Overall, these changes resulted in shorter elution times which caused some co-elution of peaks, thus it was necessary to adjust the mobile phase for further optimisation. A final isocratic mobile phase of Na-AC (50 mM, pH 4.8) / MeOH (45:55) again resulted in separation of the above mentioned peaks. The elution times for this mobile phase were daidzein, 11 min; equol, 13 min; genistein, 16 min; ODMA, 18 min. When p-ethyl-phenol (PEP) was also included in analysis, this eluted at 15 min. The new ECD system also necessitated a procedure to optimize the voltage at which analyses would be conducted. A voltammogram was established for the five isoflavone analytes of most interest (daidzein, genistein, equol, ODMA and PEP) between 500 mV and 900 mV. Based on peak area and height, optimal voltage for analysis was 750 mV or 800mV (Figure 2.1), however the background current at these two voltages were 2.2 nA and 4.9 nA, respectively. As the instructions from the manufacturer stated that the flow cell was best operated at a background current less than 3.0 nA, a voltage of 750 mV was deemed optimal. Further, the method was optimized for preferential quantification of daidzein, genistein and equol rather than ODMA or PEP.

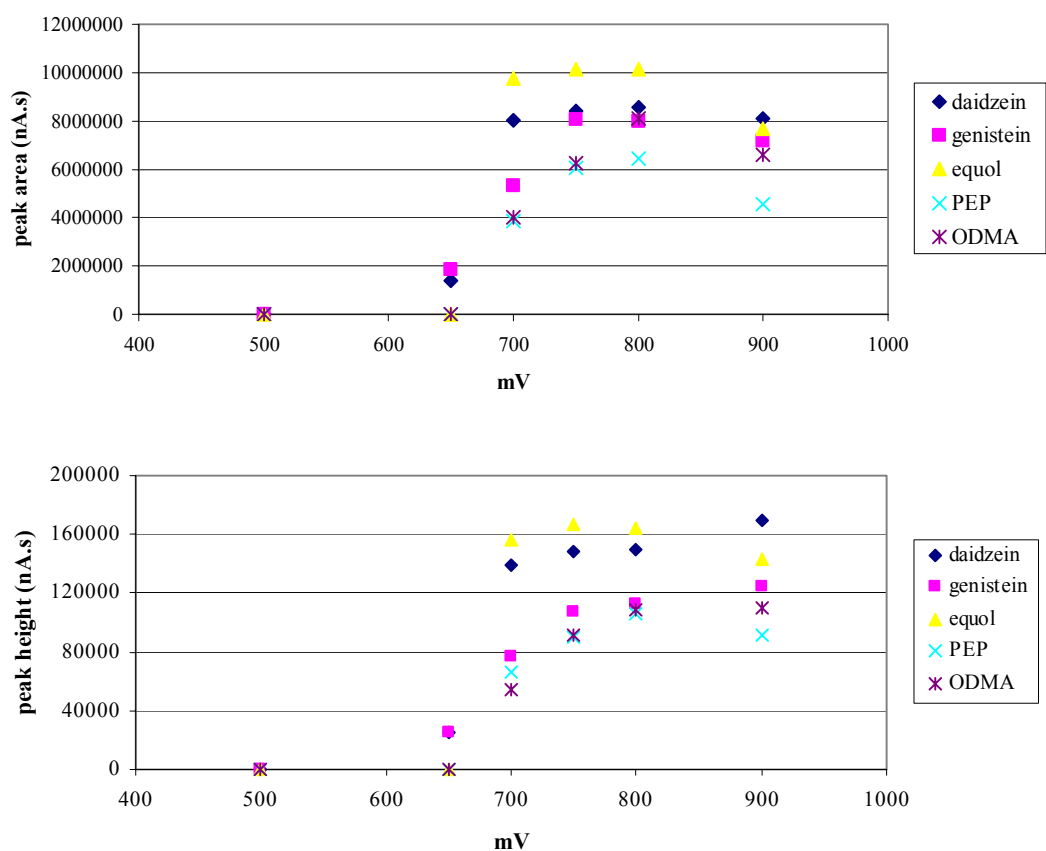


Figure 2.1 *Optimisation of Oxidative Voltages for VT-03 electrochemical flow cell for the five isoflavones investigated (100 μ M standards, 10 μ L injection).*

2.2.4 Isoflavone Recovery

In spite of the likelihood of analyte loss during extraction, few methods have used internal standards to quantify extraction recovery and at present there is no standard procedure. At the time of analysis, availability of deuterated standards was limited and their acquisition expensive, thus alternative methods of recovery were used.

Originally, a spiking recovery assay was performed in urine due to the lower levels of steroid hormones and the greater ease in obtaining urine compared with plasma. For a measure of the recovery of isoflavones in urine, the sample was first stripped of steroids

with activated charcoal and the steroid-free urine substrate was then spiked with isoflavone standards. To make activated charcoal, 10 g charcoal was added to 100 mL of water and stirred at 4°C for 1 h. Water was decanted and 1 g dextran added. Water was added to make a volume of about 100 mL, pH was adjusted to 7.4 with HCl and then made up to a final volume of 200 mL in water. This mixture of dextran charcoal was added to urine samples and gently stirred at room temperature for 2 h. The ratio of dextran: urine of 5:1 resulted in complete stripping (no peaks). Subsequently, the samples were centrifuged at 1600 g for 20 min, the supernatant removed and centrifugation was repeated. The final supernatant was filtered through a hydrophilic filter (Advantec MFS Inc, Pleasanton, CA, USA). The mean recovery was determined using 10 nmol and 20 nmol of each standard (3 of each ratio with no difference between them) and was $70 \pm 2\%$ daidzein, $78 \pm 3\%$ equol and $78 \pm 5\%$ genistein. The recoveries of ODMA and PEP were not determined due to the method not being optimal for their detection; these isoflavones were less prevalent than the other three isoflavones of greater interest.

For a simpler estimate of recovery of isoflavones in plasma, flavone (Sigma-Aldrich, Castle Hill, NSW, Australia) was used as an internal standard based on its similar structure to the isoflavones. However, flavone was not detected using the optimized HPLC conditions for isoflavone detection with a voltage of 750 mV. It was quantifiable at 800 mV, but the increased background noise with this voltage was a drawback. However, flavone can also be quantified by UV detection (Careri *et al.*, 2000) and the availability of HPLC with UV/VIS detection enabled use of this method, with minor optimisation, for flavone measurement. A mobile phase of acetonitrile / 10% acetic acid / water (80/10/10 v/v) at 1 mL/min with a C-18 column (250 x 4.6 mm, Phenomenex) and UV detection at 260 nm (SPD – 10AV VP, Shimadzu) with an injection volume of 20 μ L (SIL-10AD VP auto-injector) resulted in clear elution of flavone at 7 – 8 min. Plasma samples (200 μ L) were spiked with 0.04 nmol flavone, such that 100% recovery would equate to a final injection concentration of 20 μ M in 20 μ L, a concentration that fell in the middle of the isoflavone standard curve (standards between 0.5 μ M and 4 μ M; Figure 2.2). Mean recovery from plasma samples was $64.8 \pm 4.3\%$ (n = 80).

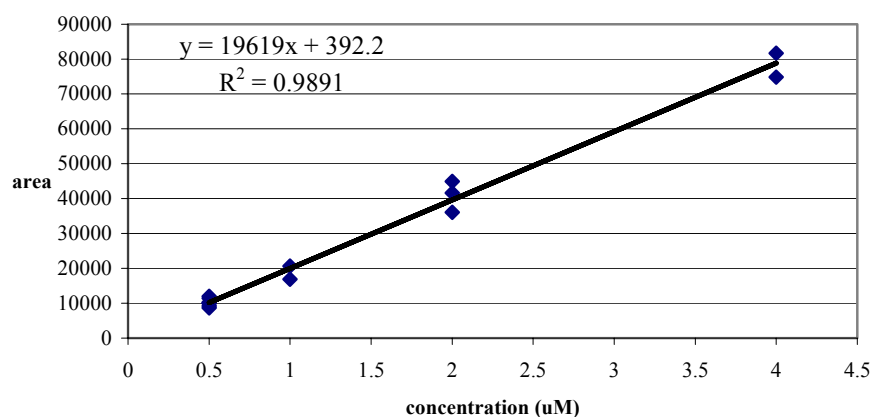


Figure 2.2 *Standard curve for flavone (UV detection at 260 nm)*

2.3 FINAL METHODS

2.3.1 Standards

All solvents used for standard preparations and extractions were HPLC grade (Crown Scientific, Moorebank, NSW, Australia). Standards of genistein (Sigma, G6649), daidzein (Sigma, D7802), equol (Sigma, 45405), O-desmethylangolensin (ODMA; Plantech, UK) and p-ethyl phenol (PEP; Plantech, UK) were made up to stocks of 2500 μM for daidzein and genistein and 2000 μM for equol, ODMA and PEP in methanol and stored at -80°C . Serial dilutions were performed to obtain concentrations of 200, 100 and 20 μM , which were also stored at -80°C . Duplicates of the 20 μM solutions were also stored at -20°C for regular daily use and further dilutions of mixed standards (all isoflavone standards combined in the one solution) were made to concentrations between 0.2 μM and 4 μM . Mixed standards of 4 different concentrations (between 0.2 μM and 2 μM for plasma and between 0.2 μM and 4 μM for urine) were included in each HPLC run at least in triplicate (at the beginning, end and in the middle of each run) but often more frequently, depending on the number of samples in a run.

Several checks were made to ensure the reliability of the isoflavone amounts reported in samples. Limits of detection (LOD) were estimated from the linear regression of the standard curves from typical runs where standards had been placed in triplicate (beginning, middle and end) in a sample series. This was done using $LOD = 3 \times \text{standard deviation (gradient)} / \text{gradient}$, which is commonly used to estimate these quantities. Limits of detection for plasma were about 10 - 15 ng/mL for daidzein and genistein, 20 - 25 ng/mL for equol and 60 - 80 ng/mL for ODMA. The corresponding values for urine were found to be 1 µg/mL daidzein and genistein, 2 – 3 µg/mL equol and 6 - 8 µg/mL ODMA. In the four studies undertaken, isoflavone values in urine were all generally well above the detection limit. For plasma though it should be noted that occasional values for daidzein, genistein and equol were close or below this nominal detection limit. These levels were included in analyses however, as there was variation in the quality of the standard curves and the signal-noise level between sets of sample runs. Nevertheless, individual isoflavone plasma values below the detection limit should be considered cautiously.

Run times for sets of samples were generally between 16 and 20 hours duration and there was high reproducibility between sets of standards in the same run. Regression equations for the standard curves were rejected if $R^2 < 0.99$ for daidzein and genistein, $R^2 < 0.975$ for equol and $R^2 < 0.90$ for ODMA and consequently, standards and samples were re-run. Example standard curves for daidzein, genistein, equol and ODMA are presented in Figure 2.3. For the four concentrations of standards used in at least triplicate in each run, the coefficients of variation (%standard deviation/mean) were less than 5% for daidzein and genistein, less than 10% for equol and less than 20% for ODMA. Within the scope of the project, sensitivity for ODMA was lower under the final conditions used and it may be concluded that detection was not optimized for ODMA. Thus levels reported in this thesis can only be considered semi-quantitative. Consequently, the occurrence of ODMA in plasma was low and there was no quantification below its limit of detection. For plasma and urine samples, the reproducibility both within and between runs was less than 10% for both daidzein and genistein; this could not be calculated for equol or ODMA due to their infrequent occurrence.

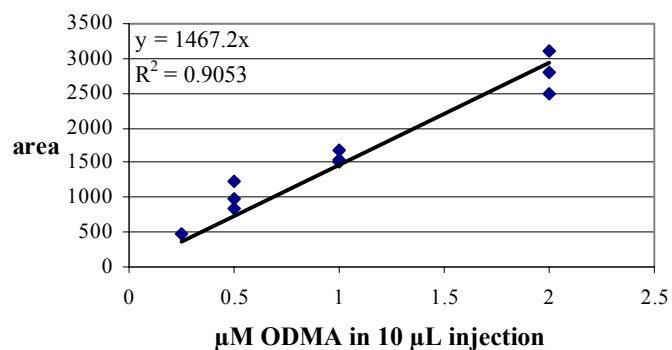
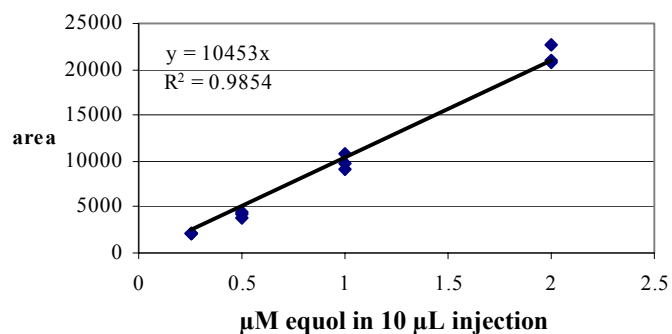
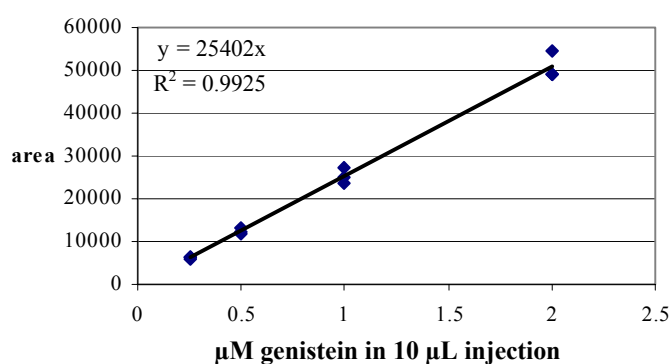
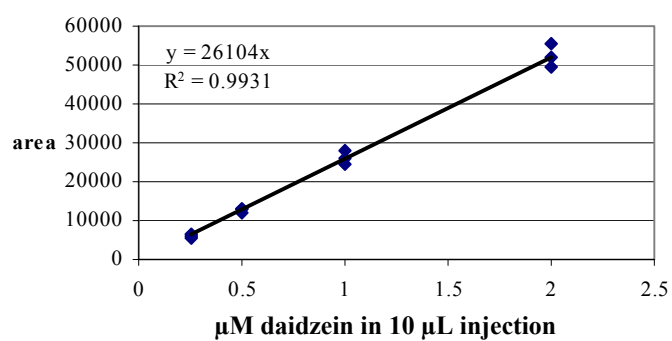


Figure 2.3 Standard curves of individual isoflavones during a run with regression lines and equations

2.3.2 Isoflavone extraction from plasma and urine

The final method for extraction of isoflavones from plasma and urine was based on that reported by Gamache and Acworth (1998) with optimizations as described above and other changes. All chemicals were from Sigma-Aldrich (Castle Hill, NSW, Australia). Samples from the studies reported in Chapter 3 and Chapter 4 were extracted by the following method. Prior to aliquoting, plasma was spun at 3500 rpm for 5 min at 4°C to remove fibrin; urine was spun at 3000 rpm for 10 min at 4°C to remove solids. An enzyme solution was made up as 5000 units/mL β -glucuronidase (Sigma #G-0751, partially purified powder from *Helix Pomatia*) in buffer (0.1 M sodium acetate containing 0.1% (w/v) ascorbic acid and 0.01% (w/v) EDTA, adjusted to pH 5.0). 200 μ L of the enzyme-buffer mix was added to 400 μ L plasma or 200 μ L urine and incubated at 37°C for 16 h with gentle shaking. Subsequently, 1200 μ L of ethanol was added to each sample which was then vortex mixed and centrifuged at 14,000 rpm for 20 min at 4°C. 1000 μ L of supernatant was collected and dried under nitrogen gas at 40°C. The extract was then re-dissolved via sonication in 400 μ L of 25% methanol. After pre-conditioning a Sep-Pak® C-18 cartridge (Waters, Rydalmere, NSW, Australia) with methanol and then distilled water, sample extracts were applied and the cartridge washed with 0.1 M sodium acetate pH 5.0 buffer (500 μ L for plasma and 1000 μ L for urine). The analyte was then eluted with methanol (500 μ L for plasma and 700 μ L for urine). This was then dried under nitrogen gas at 40°C and reconstituted in 500 μ L methanol. Samples from studies reported in Chapter 5 and Chapter 6 were extracted using the method described above, with the exception that the original aliquot of urine extracted was 50 μ L to avoid dilution prior to injection in HPLC (2.3.3).

2.3.3 HPLC separation of isoflavones and quantification with ECD

In the purified, extracted samples, isoflavones were separated on a reverse phase C-18 column (SGE Wakosil II 18RS stainless steel, 250 mm x 4.6, packing 5 μ m) using HPLC (Shimadzu auto-injector SIL-10A XL) and quantified by electrochemical detection. The electrochemical detector that was used was replaced during the course of this thesis as noted above. Thus, isoflavones from samples obtained from the first two studies were quantified slightly differently to those obtained from the later two studies, due to minor re-optimization of the separation and detection conditions for the new detector. The methods of ECD will thus be outlined separately below for the two systems used.

For samples from Chapter 3 and Chapter 4, an isocratic mobile phase of 50 mM sodium acetate buffer pH 4.8 with acetic acid / methanol / acetonitrile (42 / 50 / 8) that had been pre-filtered and pre-sonicated was used at a flow rate of 0.8 mL/min. Electrochemical detection (Coulochem II, ESA, Activon Australia) was used with a voltage of 680 mV for the analytical cell (Model 5011) with a range of 20 nA for plasma and 50 nA for urine and the conditioning cell (Model 5021) was set to 620 mV, with a range of 100 μ A to remove the signal of other compounds oxidized at this lower voltage, from the sample. Injection volume for samples (plasma and urine) into the HPLC was 10 μ L; urine was diluted 1/5 prior to injection. Peaks were identified based on the retention times of the standards in each batch and quantified from the standard curve specific to each batch using Shimadzu HPLC LC 10 analysis software. Illustration of HPLC-ECD traces of a mixed standard and of a urine sample quantified using this method are presented in Figure 2.4.

Where there was poor chromatography or an unsatisfactory signal-to-noise ratio, individual samples were re-run, with altered injection volumes if necessary. If there were any discrepancies in peak identification, the samples in question were spiked with standard to verify peaks.

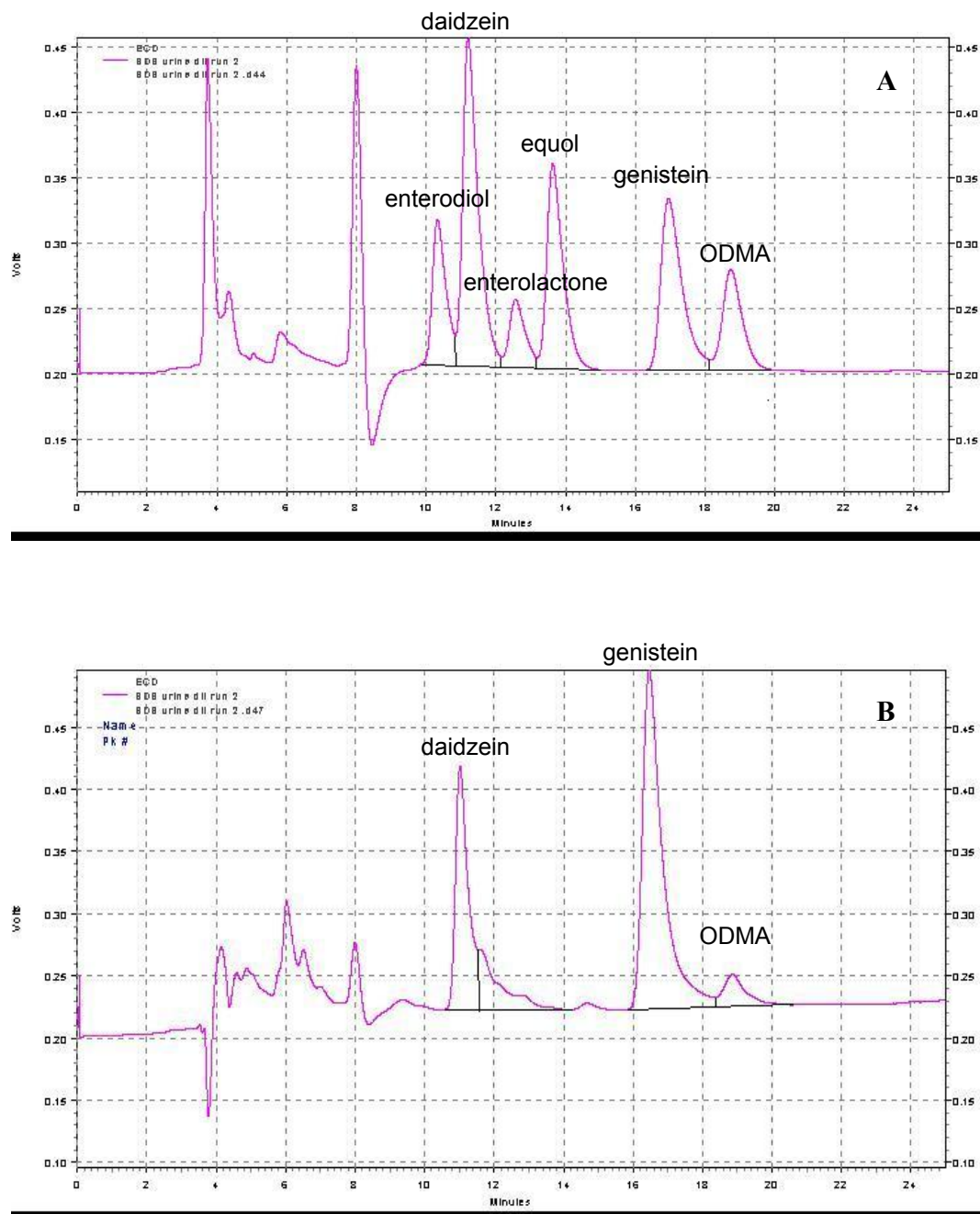


Figure 2.4 HPLC-ECD traces of *A.* mixed standard and *B.* urine sample quantified using the method used for studies reported in Chapters 3 and 4.

For samples from Chapter 5 and Chapter 6, electrochemical detection was with an electrochemical flow cell (VT-03, Antec Leyden, Zoeterwoude, The Netherlands), with the working potential set to +750 mV. An isocratic mobile phase of 50 mM sodium acetate buffer pH 4.8 with acetic acid / methanol (45 / 55) that was pre-filtered and pre-sonicated was used at a flow rate of 0.8 mL/min. Plasma and urine samples were analysed by injecting 10µL and peaks were identified based on the retention times of the standards in each batch, and quantified from the standard curve specific to each batch using Shimadzu HPLC Class VP analysis software. A HPLC-ECD trace of a sample with the trace of the middle-range standards from the same run (0.5 µM and 1.0 µM) is presented in Figure 2.6 to demonstrate the relation between the peak areas of analytes in the samples compared with the standard curve range. HPLC-ECD traces of a mixed standard and a plasma sample quantified using this method are presented in Figure 2.5.

2.3.4 Conclusions

In conclusion, this HPLC method used with electrochemical detection provided a consistent and reliable for the analysis of plasma and urinary levels of isoflavones after soy consumption. The amounts quantified are considered reliable based on routine procedures of checking the quality of chromatography and reproducibility of standards. The limits of detection are higher than those reported by Gamache and Acworth (1998) of approximately 1 ng/mL for each of daidzein, genistein and equol, however the method used in this thesis was less complicated. Further, the method used here was sufficient for analysis of plasma and urinary samples for the determination of soy intake on isoflavone bioavailability and the limits of detection reported are conservative. However, more recent developments in LC/MS analysis of isoflavones can increase the sensitivity for quantification with limits of detection for serum reported in the region of 10 pg/mL for daidzein and genistein and up to 100 pg/mL equol (Grace *et al.*, 2003).

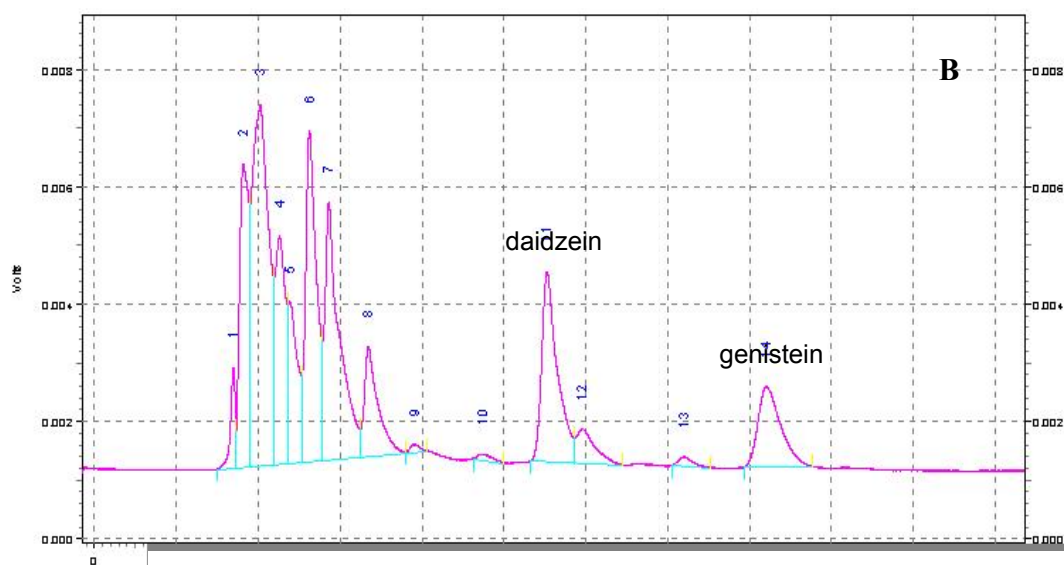
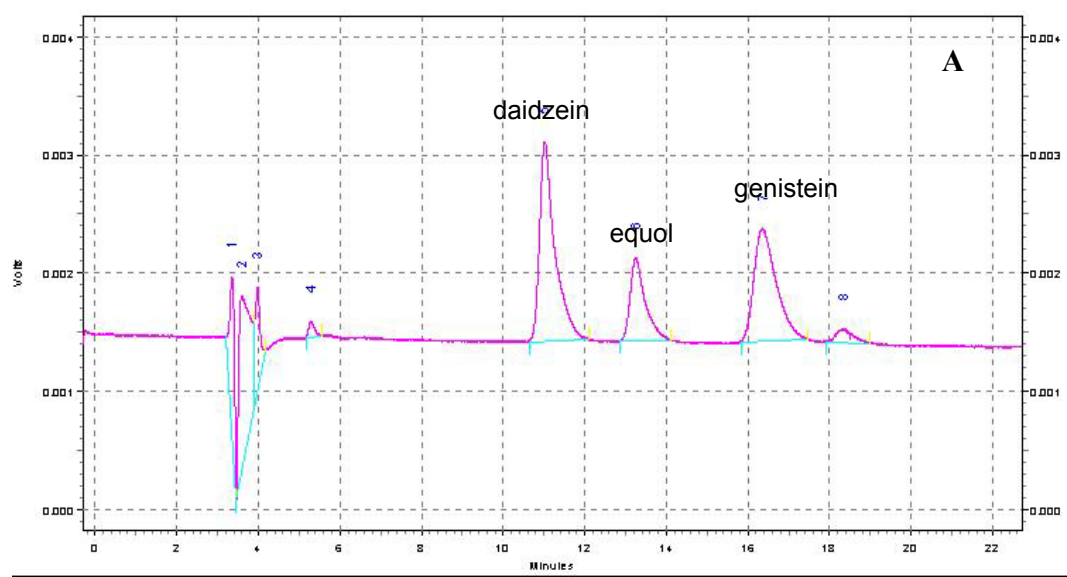


Figure 2.5 HPLC-ECD traces of **A.** mixed standard and **B.** plasma sample quantified using the method for studies reported in Chapters 5 and 6.

CHAPTER 3

EFFECT OF ACUTE AND CHRONIC RESISTANT STARCH INTAKE ON SOY ISOFLAVONE BIOAVAILABILITY IN FEMALES – A PILOT STUDY

3.1 INTRODUCTION

Soy is not a traditional ingredient in Western diets and the current average intake may be too low to produce any health beneficial effects associated with high soy consumption in Asian countries (Messina *et al.*, 1994). As the isoflavones from soy appear to play an important role in the modulation of physiological processes, enhancement of isoflavone bioavailability may be a desirable alternative to simply increasing soy food intake. The influence of many exogenous and endogenous factors on isoflavone bioavailability has direct implications for the extrapolation of results from epidemiological and clinical studies to broader populations and may explain the large inter-individual variability reported. Apart from inherent characteristics such as gender, endogenous hormone levels and gut microfloral activity, isoflavone bioavailability can be affected by the matrix in which they are contained, their relative concentrations and proportions, and interactions with other dietary components. While incorporation of soy isoflavones in typical Western foods such as breads and cereals offers more attractive choices for consumers, these matrices may affect isoflavone bioavailability. Thus, other dietary components may be an important contributor to variations in isoflavone bioavailability and diet is also one of the most important factors directly impacting on the nature and balance of the gut microflora (Wang *et al.*, 2002a).

As the absorption of many dietary constituents, including isoflavones, relies on the activity of bacteria and their enzymes in the gastrointestinal tract, much interest has

recently been directed towards identifying food components that can enhance gut microflora activity. Prebiotics are neither hydrolysed nor absorbed in the upper gastrointestinal tract and on reaching the colon, are selectively fermented by specific beneficial bacteria, thus stimulating their growth and/or activity (Gibson, 1999) and thereby producing gut microflora effects. All prebiotics are carbohydrates of relatively short chain length (Cummings *et al.*, 2001) and such dietary carbohydrates that have escaped digestion in the upper gastrointestinal tract are the principal substrates for bacterial growth (Gibson, 1998).

Starch is the main storage carbohydrate found in higher plants including cereals such as maize (Brown *et al.*, 1995); “resistant starch” is the component of starch not absorbed in the intestine and thus reaches the large intestine (Englyst *et al.*, 1996). In the colon, resistant starch (RS) provides a fermentable substrate for specific microflora, including health beneficial bifidobacteria and lactobacilli and has thus been identified as a prebiotic (Topping and Clifton, 2001). RS is easily incorporated into the diet (Brown *et al.*, 2000; Yue and Waring, 1995) and the benefits of its consumption in relation to gastrointestinal and bowel health have been promoted with its inclusion in a trademarked bread (Hi-Maize™) in Australia (Brown *et al.*, 1995). It was hypothesised in the current study that the prebiotic effects of RS may affect isoflavone bioavailability. Further, it was thought that the addition of RS to the diet might provide a more comparable gastrointestinal environment between subjects, thereby possibly minimising inter-individual variation.

This study was designed to determine whether isoflavone bioavailability after a single soy meal would be affected by (i) acute RS consumption in which this food component was consumed in the same meal as the isoflavones and (ii) chronic consumption of RS, which entailed daily RS intake for four weeks. The acute effect of RS was determined by comparing plasma and urinary isoflavones after intake of a soy meal with or without added resistant starch; while the chronic effect was determined by comparing plasma and urinary isoflavones after a soy meal consumed prior to, and two and four weeks after daily resistant starch consumption.

3.2 METHODS

3.2.1 Subjects

Pre-menopausal, omnivorous, female volunteers in good general health were recruited from the University of Wollongong. Exclusion criteria included a history of gastrointestinal pathology, use of antibiotics, laxatives or prebiotics in the previous 3 months and intake of more than two standard alcoholic drinks per day. Approval for the study was obtained from the University of Wollongong Human Ethics Committee (Ethics Approval Number HE 99/165) and all subjects signed informed consent forms. Ten subjects commenced the study.

3.2.2 Study design

Three weeks prior to commencement of the study, subjects were instructed to remove all foods of high phytoestrogen or RS content as well as any sources of probiotic cultures from their diets until the study was completed. Apart from these requirements, and the inclusion of study foods, subjects maintained their usual diets for the duration of the study. Study design is summarised in Figure 3.1. After the initial 3-week wash-in period, subjects consumed a control soy meal (soy cereal and soy milk) as breakfast between 6:30 and 8:00 am after fasting overnight. Blood and urine samples were collected 5, 7, 9, and 11 h post-meal and a 12 h urine collection commenced after the 11 h sample. Six days later, the acute effect of RS was determined by consuming the same soy meal, but with the addition of 20 g raw RS sprinkled on the cereal. Blood and urine were collected at the same post-meal times. The next day, subjects commenced chronic RS intake, incorporating 20 g RS per day into their diet. After 2 and 4 weeks of daily RS intake, subjects again consumed the soy meal (without RS) and blood and urine were sampled as per the original soy meal (Figure 3.1).

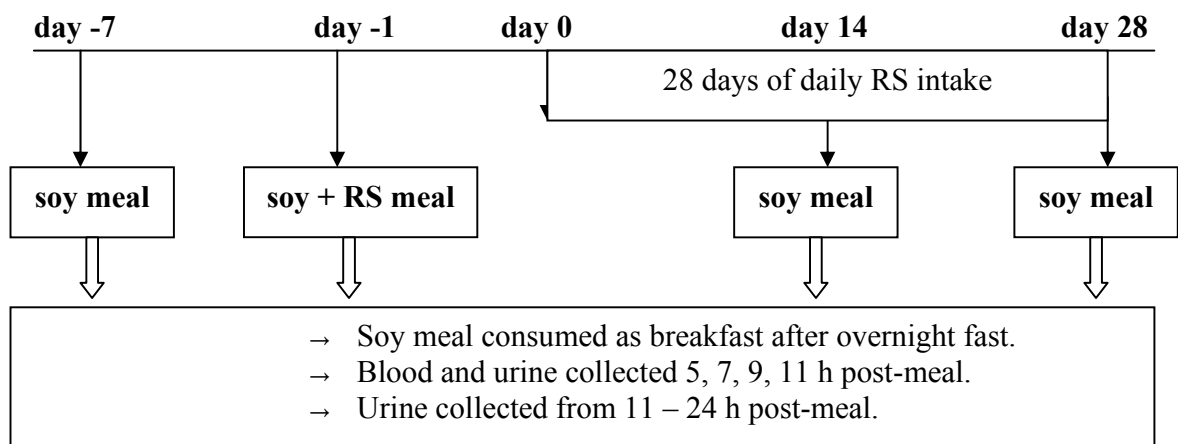


Figure 3.1 Study Design

3.2.3 Study foods

For each soy meal, subjects consumed 45 g of soy-enriched cereal (Soy and Fibre Ultra-Bran, Kellogg's, Botany, NSW, Australia) providing approximately 45 mg of total isoflavones (aglycone units) with 250 mL of soy milk (So Good, Sanitarium Health Food Company, Berkeley Vale, NSW, Australia) providing 20 mg of total isoflavones (aglycone units). The same lot number of cereal and milk were purchased to minimize variability in isoflavone levels. Subjects were provided with these foods the day prior to consumption and were instructed to consume the complete amount of pre-weighed soy cereal, but were responsible for measuring out the set volume of soy milk themselves. The resistant starch (RS) was provided in the form of high amylose (70%) flour (Product #1076, Penford Australia Ltd, Lane Cove, NSW, Australia) and was pre-weighed into 29 g packages, each providing 20 g RS. This was given to subjects the day prior to the acute RS meal and weekly during the chronic RS stage. The high amylose maize starch used could be added to foods such as breads and breakfast cereals and to milk and non-milk drinks (Brown *et al.*, 2000; Yue and Waring, 1995), and due to its high gelatinization temperature, can survive most normal food processing conditions (Brown, 1996). Subjects were provided advice on how to include this supplement in their background diets, with suggestions of mixing it in milk or juice, or with cereals or stews.

3.2.4 Sample collection

Blood collection was carried out by a registered nurse in the Clinical Area of the Department of Biomedical Science, at the University of Wollongong. Blood (7.5 mL) was collected into EDTA tubes (Sarstedt) and after inversion the tubes were placed on ice until the end of each collection period (approximately 1 h). Following this, blood was spun at 4°C for 10 min at 3500 rpm in a refrigerated centrifuge (Hettich, Universal 16R), plasma was harvested and aliquots were stored at -20°C until analysis. Spot urine samples were collected from each subject in sample collection vials and immediately placed in the fridge. After each collection period, 5 mL aliquots were stored at -20°C with 450 µL sodium azide (1%) until analysis. The overnight urine samples were collected in 2.5 L collection bottles that contained 1 g ascorbic acid. After measurement of the volume of each overnight sample, aliquots were stored in the same way as for the spot samples.

3.2.5 Isoflavone analysis of plasma and urine

Isoflavones were extracted from plasma and urine as described in 2.3.2 and quantified by HPLC and ECD as per 2.3.3.

3.2.6 Statistical analysis

Initially, distribution of data was determined and outliers were identified (SPSS 11.0 and 11.5, SPSS Inc. Chicago, Illinois). Paired Student's t-tests (Microsoft Excel, Microsoft Corporation) and ANOVA with repeated measures (ANOVA/RM; SPSS 11.0 and 11.5, SPSS Inc. Chicago, Illinois) and Bonferroni post-hoc analysis were used wherever possible. When post-hoc tests were not automatically included in the results of ANOVA, Bonferroni correction was used with the significance level adjusted for multiple t-tests. Correlations are reported as Pearson's Correlation coefficient (R).

3.3 RESULTS

3.3.1 Subjects and compliance

Ten subjects (8 Caucasian and 2 Kenyan) commenced this study, but not all subjects completed all components. One subject was eliminated from the final analyses because they only participated in the acute RS component and after the soy + RS meal, their plasma isoflavone levels were outliers at all 4 time points compared with these values for the other subjects. One subject was unavailable for the soy + RS meal, and three subjects withdrew from the study after completing 2 weeks of the chronic RS component, due to other commitments for two subjects, whilst the third withdrew due to their dislike of the RS additive. Otherwise the study foods were tolerated, however subjects commonly reported minor gastrointestinal side-effects including mild bloating and extra flatulence. Details for the nine subjects who completed most parts of the study are presented below (Table 3.1). The number of subjects for whom data were included in analyses is reported in each section. One subject did not provide urine at any time.

Table 3.1 Subject Characteristics (n = 9).

	age (years)	weight (kg)	height (cm)	BMI (kg/m²)
mean ± SEM	21.3 ± 0.7	62.1 ± 2.7	166.8 ± 1.7	22.3 ± 0.7
range	18.5 - 24	48 - 73.8	161.2 - 174	18.2 - 24.4

3.3.2 Baseline isoflavones

As expected, the baseline plasma samples of the 9 subjects obtained prior to the control soy meal, contained little or no isoflavones (Figure 3.2). One subject had low levels of both daidzein and genistein in their plasma (39.5 ng/mL and 56.5 ng/mL respectively), while two others had genistein only (26.0 ng/mL and 52.7 ng/mL). These were still less than the lowest plasma concentrations obtained after the control soy meal (52.0 ng/mL daidzein and 100 ng/mL genistein).

3.3.3 Plasma isoflavones after control soy meal

After consumption of the control soy meal, daidzein and genistein were present in the plasma of all subjects ($n = 9$), but with large inter-individual variation (Figure 3.2). There were significant differences over time for both daidzein and genistein when baseline samples were included in the analyses ($F_{4,4} = 11.569$, $p = 0.018$ and $F_{4,4} = 19.639$, $p = 0.007$ respectively), but not between the four post-meal samples ($p = 1.000$ for both daidzein and genistein, one-way ANOVA/RM and Bonferroni post-hoc analysis, Figure 3.2). Daidzein and genistein showed a similar pattern of plasma absorption across the post-meal samples, both with a peak mean plasma concentration at 7 h post-meal, although not statistically significant ($p = 1.000$ for both daidzein and genistein); 125 ± 17.2 ng/mL daidzein and 278 ± 61.6 ng/mL genistein. The plasma concentration of genistein was significantly greater than that of daidzein ($F_{1,7} = 21.063$, $p = 0.003$, two-way ANOVA/RM) with the mean level of genistein approximately double that of daidzein at all times.

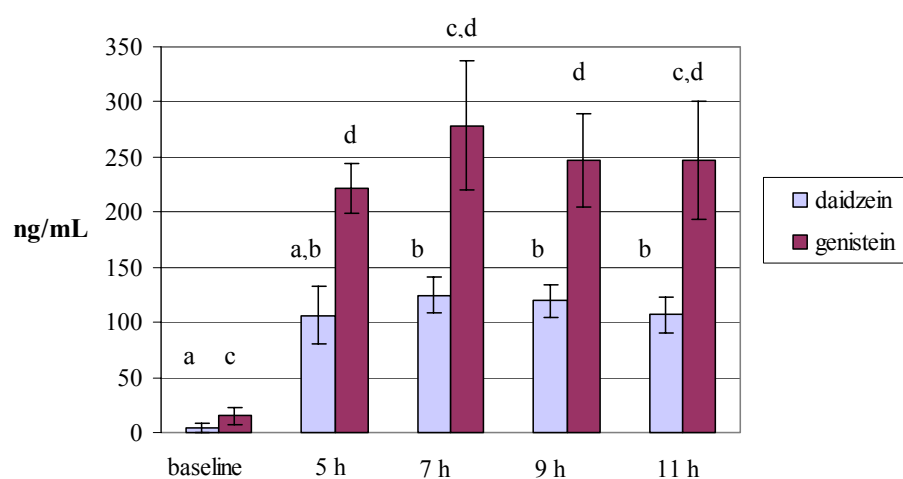


Figure 3.2 Mean plasma isoflavone levels after control soy meal. Mean \pm SEM, $n=9$. Values with different letters are significantly different ($p < 0.05$, Bonferroni post-hoc analysis).

The maximum concentration (C-max) of plasma daidzein occurred at 7 or 9 h post-meal for 6 of the 9 subjects and for genistein at 5 or 7 h post-meal for 7 subjects. The mean maximum plasma isoflavone concentrations were 178 ± 23.1 ng/mL daidzein (range 85 - 315 ng/mL) and 355 ± 62.3 ng/mL genistein (range 164 – 710 ng/mL). There was high correlation between plasma daidzein and genistein for individuals ($R = 0.663$, $p < 0.01$, $n = 35$) when all post-meal samples were included.

As an estimate of overall plasma bioavailability for the 11 h sampling period, the “area under the curve” (AUC) was calculated for each individual. This was the sum of the areas formed beneath the five sample values, when baseline was included as 0 h (example, Figure 3.3). The mean AUC after the control soy meal was 968 ± 99 ngmL⁻¹h daidzein (range 643 – 1611 ngmL⁻¹h) and 2049 ± 222 ngmL⁻¹h genistein (range 1118 – 3159 ngmL⁻¹h). The AUC of genistein was significantly higher than that of daidzein ($p = 0.005$, Student’s paired t-test) and the average daidzein: genistein ratio (D/G) for AUC was 0.51 ± 0.07 (range 0.29 – 0.81).

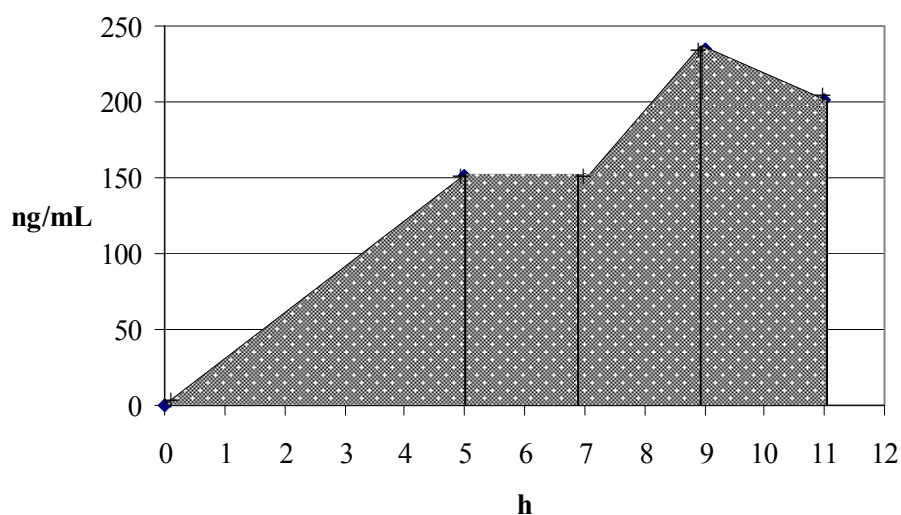


Figure 3.3 Sample AUC representation. Subject 2, plasma genistein after 2 weeks RS. Calculated AUC = 1498.2 ngmL⁻¹h.

3.3.4 Urinary isoflavones after control soy meal

Urinary isoflavone levels for the spot samples after each of the 4 test meals are presented as $\mu\text{g/mL}$, as total excretion was not recorded for these samples. All values presented here are greater than the limits of detection (also expressed as $\mu\text{g/mL}$ urine) reported in Chapter 2. After the control soy meal, all subjects excreted daidzein and genistein in their urine from as early as the 5 h sample, but with considerable variation between subjects (Figure 3.4). There were no significant differences between the 4 post-meal spot urine samples for either isoflavone ($F_{3,5} = 0.888$, $p = 0.508$ for daidzein, $F_{3,5} = 0.550$, $p = 0.670$ for genistein, one-way ANOVA/RM) nor between the urinary concentrations of daidzein and genistein ($F_{1,7} = 2.005$, $p = 0.200$, two-way ANOVA/RM). When individual values were averaged for each time point, there was a trend for both daidzein and genistein to be at their greatest mean concentration 7 h post-meal; $7.8 \pm 3.0 \mu\text{g/mL}$ and $7.6 \pm 3.4 \mu\text{g/mL}$ respectively. The greatest mean increase for both isoflavones was between 5 and 7 h, after which there was a steady decline in their concentrations.

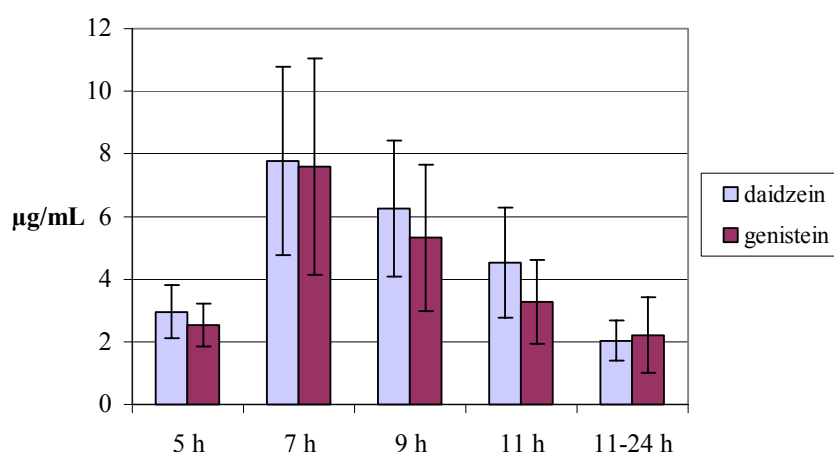


Figure 3.4 Mean urinary isoflavone levels after the control soy meal. Mean \pm SEM, $n=8$.

Although the maximum mean urinary excretion was at 7 h for both daidzein and genistein, the majority of subjects had their greatest urinary concentration of daidzein at either 9 or 11 h post-meal (6 of 8 subjects) and of genistein at either 5 or 7 h post-meal (7 of 8 subjects). This highlights the large inter-individual variation. The mean C-max for all subjects was 9.9 ± 3.0 $\mu\text{g/mL}$ daidzein (range 1.5 – 26.3 $\mu\text{g/mL}$) and 8.6 ± 3.4 $\mu\text{g/mL}$ genistein (range 0.81 – 28.5 $\mu\text{g/mL}$). To determine the total amount excreted overnight, the overnight concentration was adjusted for urine volume for each individual. The average total amount excreted 11 – 24 h post-meal was 1.1 ± 0.27 mg daidzein (range 0.3 – 2.3 mg) and 0.94 ± 0.30 mg genistein (range 0.19 – 2.2 mg). There were no significant differences between daidzein and genistein overnight excretion for concentration or total amount ($p = 0.857$ and $p = 0.790$, respectively, Student's paired t-tests).

The mean urinary AUC values between 5 and 11 h were 35.5 ± 10.3 $\mu\text{gmL}^{-1}\text{h}$ daidzein (range 8.1 – 97.0 $\mu\text{gmL}^{-1}\text{h}$) and 31.6 ± 11.9 $\mu\text{gmL}^{-1}\text{h}$ genistein (range 3.6 – 103.3 $\mu\text{gmL}^{-1}\text{h}$). There was no significant difference between these values ($p = 0.288$, Student's paired t-test) and the mean D/G ratio for AUC was 1.56 ± 0.24 . The concentrations of daidzein and genistein in individual urine samples were similar and highly correlated ($R = 0.995$, $p < 0.001$, $n = 32$).

3.3.5 Effects of acute RS intake on plasma and urinary isoflavones

The addition of RS to the control soy meal significantly reduced the plasma daidzein and genistein concentration of post-meal samples ($n = 8$, $F_{1,4} = 14.96$, $p = 0.018$ and $F_{1,4} = 7.36$, $p = 0.053$, respectively, two-way ANOVA/RM, Figure 3.5) and C-max and AUC for both isoflavones (Table 3.2). Again, there were no significant differences between the 4 post-meal samples for either isoflavone ($F_{3,3} = 3.047$, $p = 0.192$ for daidzein and $F_{3,3} = 0.753$, $p = 0.590$ for genistein, one-way ANOVA/RM). The plasma level of genistein was significantly higher than that of daidzein ($F_{1,5} = 10.727$, $p = 0.022$, two-way ANOVA/RM) as were C-max and AUC (Table 3.2). There was high correlation between daidzein and genistein in all post-meal samples ($R = 0.708$, $p = 0.01$, $n = 30$).

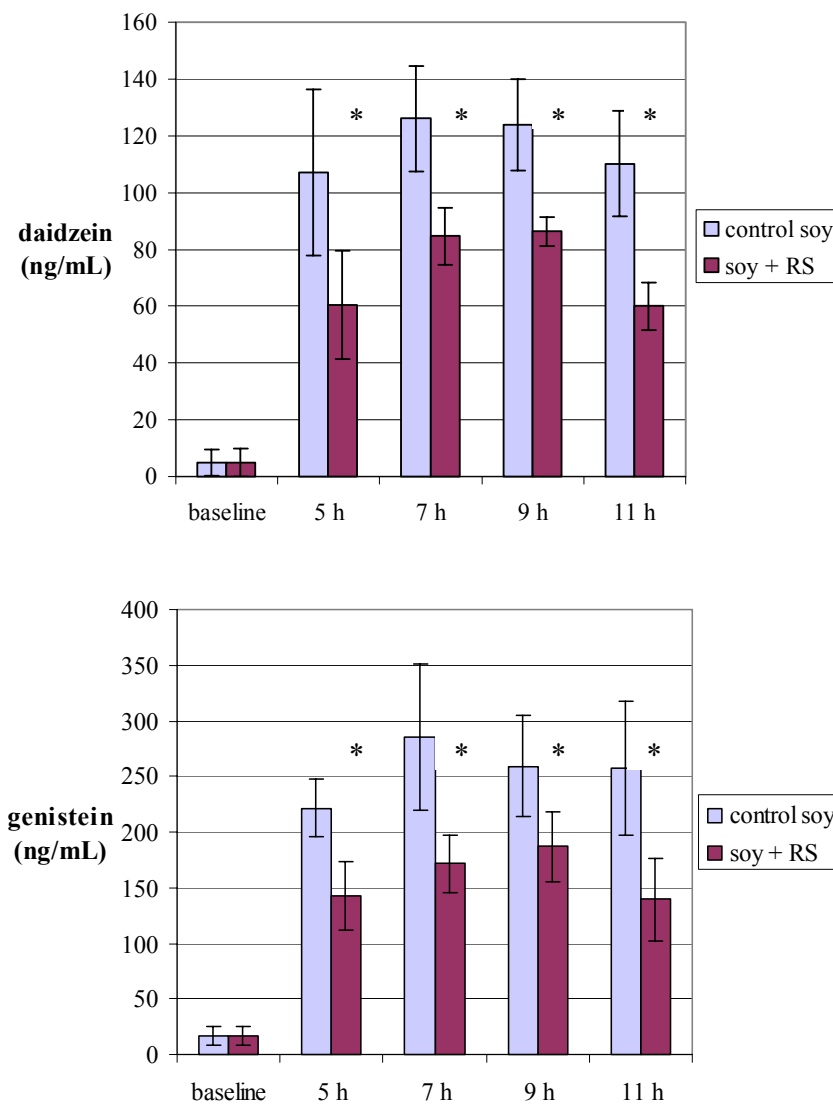


Figure 3.5 Effects of acute RS on mean plasma daidzein and genistein. Mean \pm SEM, n=8. *Significantly different compared with control soy meal, two-way ANOVA with repeated measures.

In contrast to the significant reduction in plasma isoflavones, there were no significant differences in urinary isoflavone levels after the soy + RS meal compared with the control soy meal for spot samples (n = 7, $F_{1,6} = 1.721$, $p = 0.237$ for daidzein and $F_{1,6} = 3.489$, $p = 0.111$ for genistein, two-way ANOVA/RM), C-max, AUC or total amount

excreted overnight (Table 3.2). There were also no differences between any of the post-meal samples for either daidzein or genistein ($F_{3,4} = 0.339$, $p = 0.800$ and $F_{3,4} = 1.025$, $p = 0.470$ respectively, one-way ANOVA/RM). When the means were compared, there was a trend for daidzein and genistein at 7 and 9 h to be reduced by acute RS, although this was not statistically significant (Figure 3.6). There was no significant difference between the urinary concentrations of daidzein and genistein after the soy + RS meal ($F_{1,6} = 2.709$, $p = 0.151$, two-way ANOVA/RM) and again there was high correlation between daidzein and genistein ($R = 0.483$, $p = 0.01$, $n = 28$).

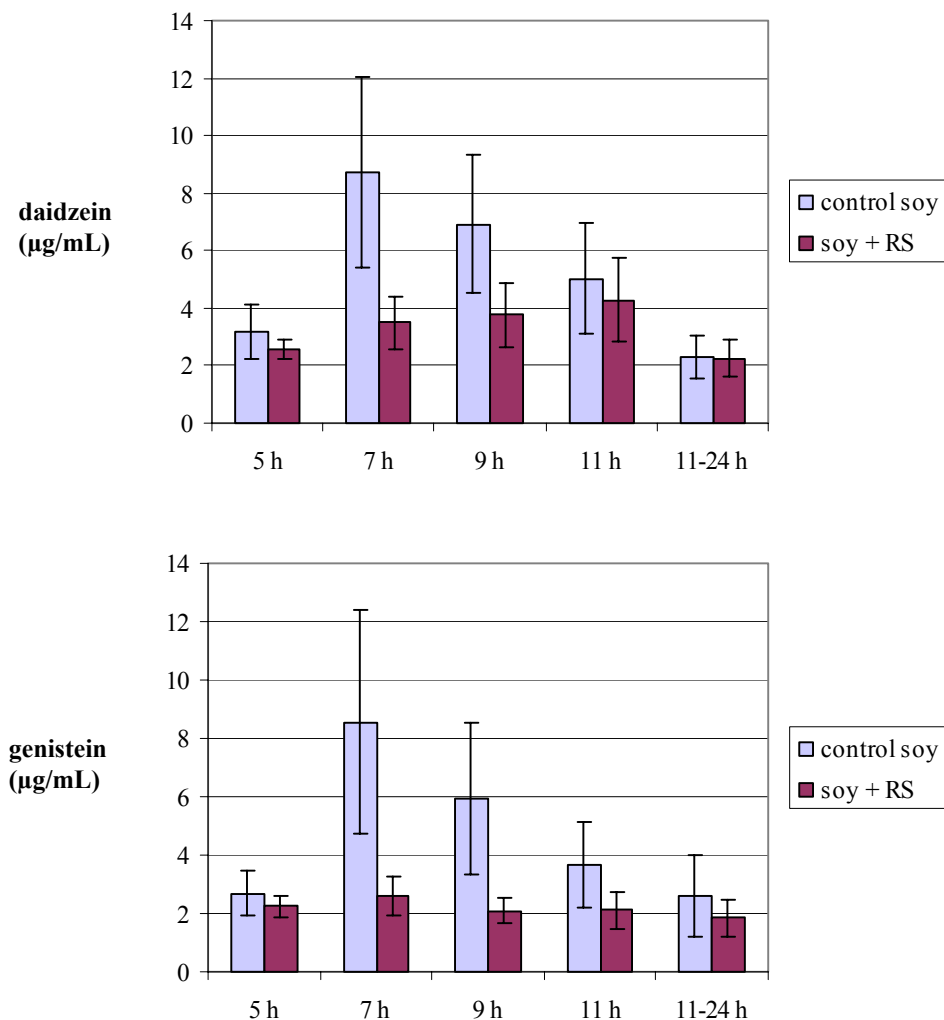


Figure 3.6 Effects of acute RS on mean urinary daidzein and genistein. Mean \pm SEM, $n=7$.

Table 3.2 Comparison of effects of control soy meal vs. soy + RS meal on plasma and urinary parameters. Mean \pm SEM, range in parentheses. O/N = overnight.

		control soy			soy + RS [†]		
		daidzein	genistein	p [‡]	daidzein	genistein	p [‡]
Plasma	C-max (ng/mL)	186 \pm 24.6 (85 – 315)	371 \pm 68.5 (164 – 710)	0.019	111 \pm 12.9 * (74 – 189)	229 \pm 28.1 ** (114 – 337)	0.002
	AUC (ngmL ⁻¹ h)	986 \pm 144 (643 – 1611)	2090 \pm 320 (1118 – 3159)	0.004	626 \pm 68 * (431 – 1032)	1380 \pm 173 ** (790 – 1983)	0.001
Urine	Cmax (μ g/mL)	11.1 \pm 3.2 (2.3 – 26.3)	9.7 \pm 3.7 (0.81 – 28.5)	0.202	5.1 \pm 1.3 (2.2 – 11.9)	3.4 \pm 0.48 (2.3 – 5.6)	0.546
	AUC (μ gmL ⁻¹ h)	39.5 \pm 11.0 (9.3 – 97.0)	35.3 \pm 13.1 (3.6 – 103.3)	0.331	21.3 \pm 5.5 (5.3 – 38.2)	13.7 \pm 2.8 (1.0 – 25.9)	0.150
	O/N (mg)	1.20 \pm 0.24 (0.7 – 2.3)	1.09 \pm 0.27 (0.39 – 2.2)	0.792	1.13 \pm 0.22 (0.46 – 1.72)	0.97 \pm 0.28 (0.13 – 2.03)	0.532

[†] Values with asterisk are significantly different compared with control soy meal (* p < 0.05, ** p < 0.01, Student's paired t-tests).

[‡] Student's paired t-tests for comparison between daidzein and genistein.

3.3.6 Effects of 2 weeks RS intake on plasma and urinary isoflavones

During chronic RS intake, soy meals as per the control soy meal (without RS added) were consumed on day 14 and 28. The effect of 2 weeks RS was analysed initially as all subjects completed this component. Although there were no overall significant effects of 2 weeks RS intake on plasma daidzein or genistein, there was a trend toward a reduction in these levels (n = 9, daidzein: $F_{1,4} = 2.699$, p = 0.176 and genistein: $F_{1,5} = 2.908$, p = 0.149, two-way ANOVA/RM, Figure 3.7). When only the 5, 9 and 11 h samples were analysed (as multiple subjects missed the 7 h sample after either meal), there was a significant reduction in plasma daidzein after 2 weeks RS intake but no effect on plasma genistein ($F_{1,6} = 8.507$, p = 0.027 and $F_{1,7} = 2.818$, p = 0.137 respectively, two-way ANOVA/RM, Figure 3.7).

There were no significant differences between the post-meal samples for either isoflavone ($F_{3,3} = 1.923$, p = 0.302 for daidzein and $F_{3,4} = 2.073$, p = 0.247 for genistein, one-way ANOVA/RM) and plasma genistein was again significantly higher than plasma daidzein ($F_{1,5} = 37.541$, p = 0.002, two-way ANOVA/RM). Although reduced, the

overall patterns for both isoflavones were not altered in that the mean concentrations at 7 h and 9 h were more elevated than at 5 h and 11 h. The mean C-max and AUC were also reduced after 2 weeks RS for both daidzein and genistein, although this was only significant for the former (Table 3.3). Plasma daidzein and genistein concentrations were highly correlated per individual ($R = 0.841$, $n = 33$, $p = 0.01$).

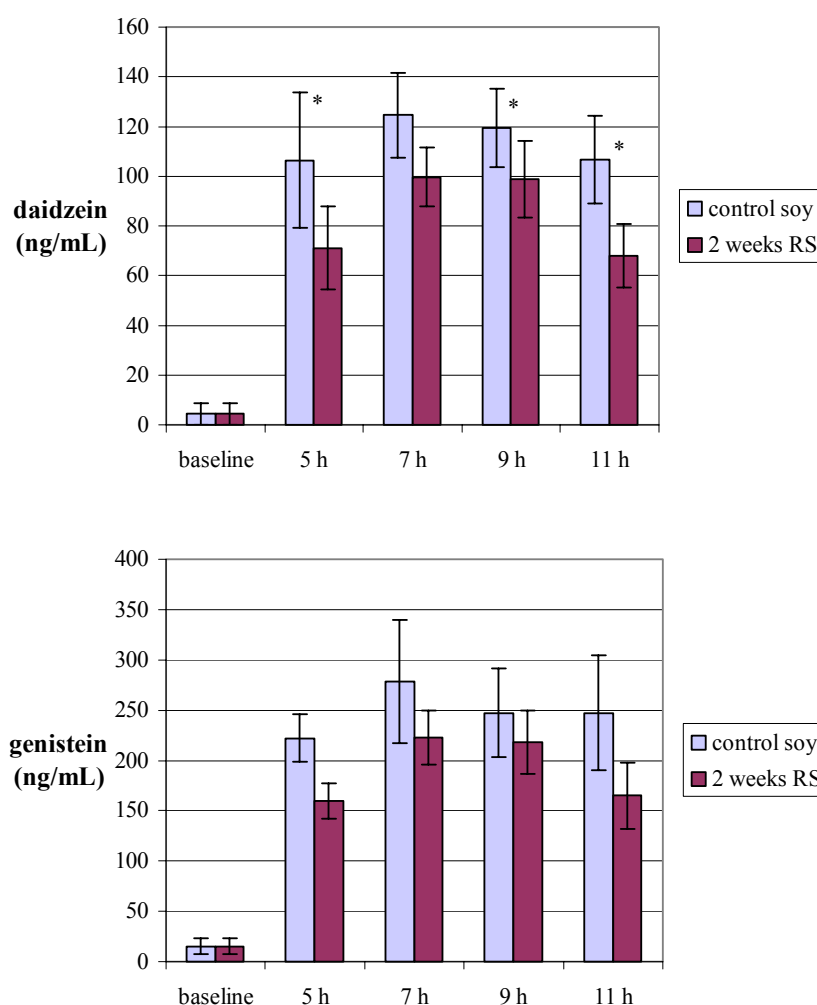


Figure 3.7 Mean plasma daidzein and genistein following soy meal before and after 2 weeks RS intake. Mean \pm SEM, $n=9$. *Significantly different compared with control soy meal, two-way ANOVA with repeated measures.

Table 3.3 Comparison between control soy vs. 2 weeks RS on plasma and urinary parameters. Mean \pm SEM, range in parentheses. O/N = overnight.

		control soy		p^{\dagger}	2 weeks RS [†]		p^{\dagger}
		daidzein	genistein		daidzein	genistein	
Plasma	C-max (ng/mL)	178 \pm 23.1 (85 – 315)	355 \pm 62.3 (164 – 710)	0.011	122 \pm 9.2 * (70 – 161)	255 \pm 21.5 (192 – 408)	<0.001
	AUC (ngmL ⁻¹ h)	968 \pm 99 (643 – 1611)	2049 \pm 222 (1118 – 3159)	0.002	692 \pm 88 * (223 – 1191)	1582 \pm 162 (1133 – 2787)	<0.001
Urine	C-max (μ g/mL)	9.9 \pm 3.0 (1.5 – 26.3)	8.6 \pm 3.4 (0.81 – 28.5)	0.197	12.7 \pm 2.8 (1.7 – 24.0)	8.45 \pm 2.06 (1.2 – 17.1)	0.094
	AUC (μ gmL ⁻¹ h)	35.5 \pm 10.3 (8.1 – 97.0)	31.6 \pm 11.9 (3.6 – 103.3)	0.288	39.9 \pm 7.7 (8.3 – 66.6)	28.6 \pm 6.3 (3.7 – 56.1)	0.087
	O/N (mg)	1.05 \pm 0.27 (0.33 – 2.3)	0.94 \pm 0.30 (0.19 – 2.2)	0.792	2.6 \pm 1.1 (0.41 – 6.7)	1.14 \pm 0.53 (0.13 – 2.03)	0.532

[†] Values with asterisk are significantly different compared with control soy meal (* $p < 0.05$, ** $p < 0.01$, Student's paired t-tests).

[‡] Student's paired t-tests for comparison between daidzein and genistein.

There were no significant effects of 2 weeks RS intake on the urinary concentrations of either daidzein or genistein ($F_{1,7} = 0.212$, $p = 0.659$ and $F_{1,7} = 0.000$, $p = 0.987$ respectively, two-way ANOVA/RM, Figure 3.8). However, there was a trend that approached significance for urinary daidzein concentration to be greater than that of genistein ($F_{1,7} = 4.817$, $p = 0.064$, two-way ANOVA/RM). Although there were no significant differences between the post-meal samples ($F_{3,5} = 0.703$, $p = 0.590$ for daidzein and $F_{3,5} = 0.810$, $p = 0.541$ for genistein, one-way ANOVA/RM), the highest mean concentrations were both at 9 h, 7.38 ± 2.51 μ g/mL daidzein and 5.29 ± 2.01 μ g/mL genistein, suggesting a delay in urinary excretion of the isoflavones compared with the control soy meal. In addition, the daidzein concentration of the overnight urine sample tended to be greater than after the control soy meal, with a mean of 4.35 ± 1.5 μ g/mL compared with 2.36 ± 0.68 μ g/mL ($p = 0.096$, Student's paired t-test). Daidzein and genistein levels were again significantly correlated ($R = 0.777$, $p = 0.01$, $n = 31$).

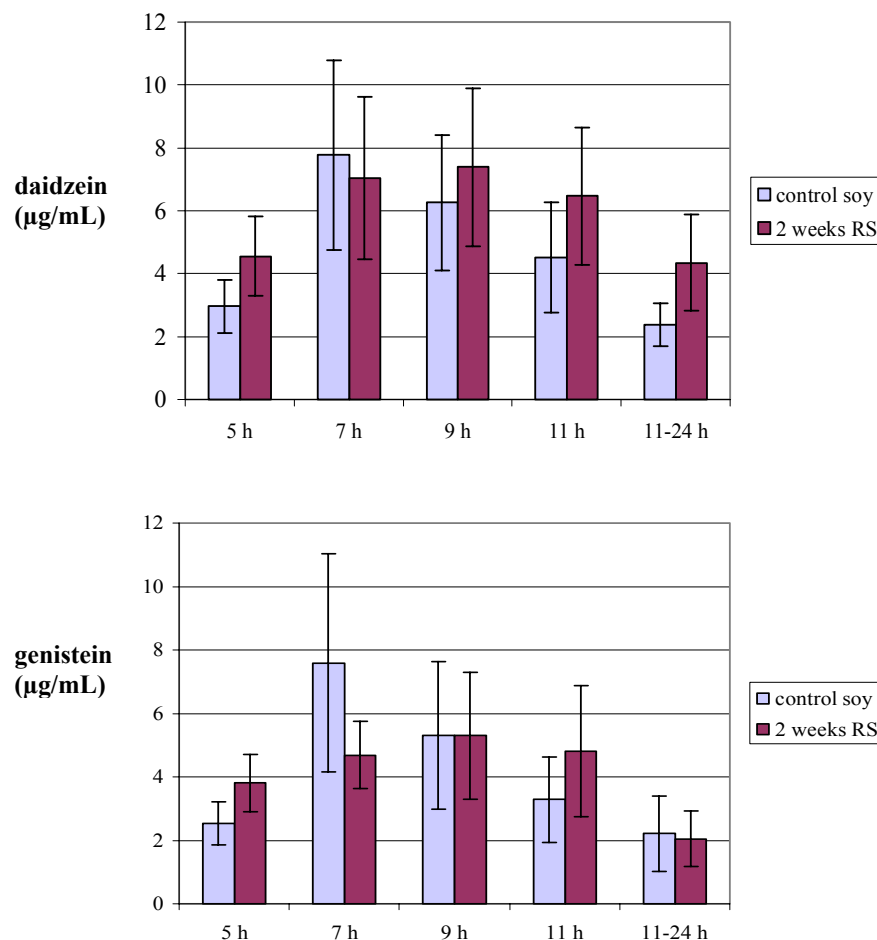


Figure 3.8 Mean urinary daidzein and genistein following soy meal before and after 2 weeks RS intake. Mean \pm SEM, n=8.

3.3.7 Effects of 4 weeks RS intake on plasma and urinary isoflavones

For determination of the effects of 2 and 4 weeks chronic RS intake, only the subjects who completed 4 weeks chronic RS intake were included to maintain paired and repeated measures analyses (n = 6 for plasma; n = 5 for urine). Removal of the data of the three subjects who did not complete 4 weeks of daily RS intake did not significantly alter the mean plasma or urinary daidzein or genistein concentrations following the control soy meal or the soy meal after 2 weeks of RS intake (Appendix 3.1).

For those subjects who completed 2 and 4 weeks RS intake, plasma daidzein and genistein did not differ significantly between the three soy meals ($n = 6$, $F_{2,2} = 1.859$, $p = 0.350$ and $F_{2,2} = 0.808$, $p = 0.553$, respectively, two-way ANOVA/RM) nor between control and 4 weeks RS ($F_{1,4} = 4.522$, $p = 0.101$ and $F_{1,4} = 2.894$, $p = 0.164$, respectively, two-way ANOVA/RM, Figure 3.8). After 4 weeks RS, there were no significant differences between post-meal samples for either daidzein or genistein ($F_{3,2} = 0.612$, $p = 0.669$ and $F_{3,2} = 1.835$, $p = 0.372$, respectively, one-way ANOVA/RM) and the plasma concentration of genistein was again significantly higher than of daidzein ($F_{1,4} = 27.735$, $p = 0.006$, two-way ANOVA/RM). Plasma daidzein and genistein concentrations were again correlated per subject ($R = 0.491$, $p = 0.017$, $n = 23$).

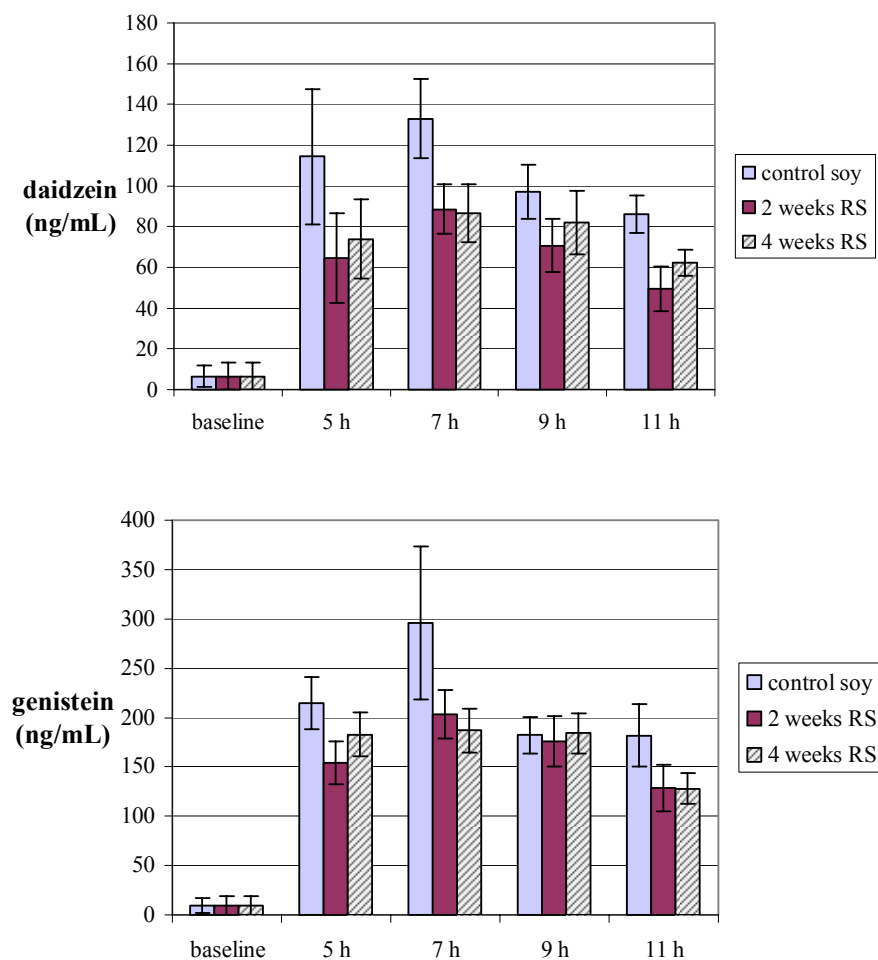


Figure 3.9 Mean plasma daidzein and genistein following soy meals during 4 weeks chronic RS intake. Mean \pm SEM, $n=6$.

There were no significant effects of chronic RS intake on the urinary excretion of daidzein or genistein ($F_{2,3} = 0.047$, $p = 0.955$ and $F_{2,3} = 0.037$, $p = 0.964$, respectively, two-way ANOVA/RM, Figure 3.10). After 4 weeks RS, the trend for urinary daidzein to be higher than genistein again approached significance ($F_{1,4} = 5.380$, $p = 0.081$, two-way ANOVA/RM) and there were no differences between any of the post-meal samples ($F_{3,2} = 1.833$, $p = 0.372$ for daidzein and $F_{3,2} = 0.660$, $p = 0.649$ for genistein, one-way ANOVA/RM). There was however, a significant increase in the amount of daidzein excreted overnight after 4 weeks RS intake, 1.57 ± 0.54 mg compared with 1.12 ± 0.60 mg after the control soy meal ($p = 0.017$, Student's paired t-test). In contrast, the amount of genistein excreted after 4 weeks RS intake was similar to that after the control soy meal, 0.77 ± 0.20 mg compared with 0.78 ± 0.36 mg ($p = 0.984$, Student's paired t-test).

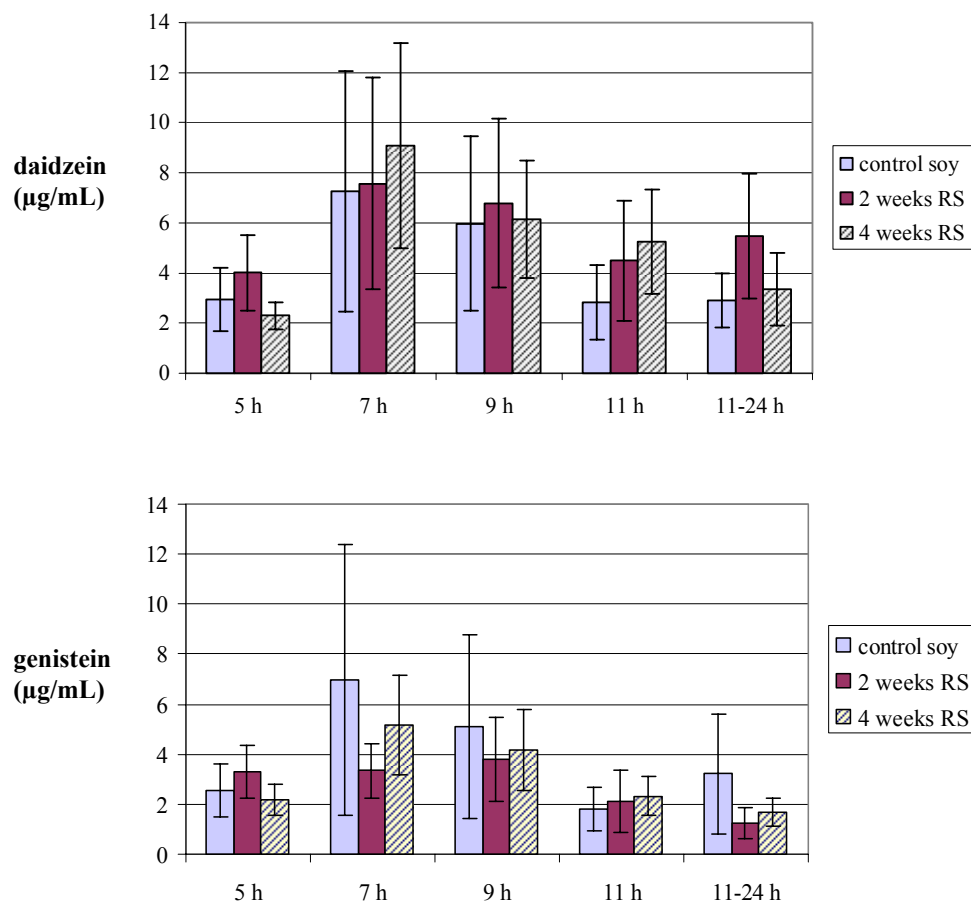


Figure 3.10 Mean urinary daidzein and genistein during chronic RS intake. Mean \pm SEM, $n = 5$.

There were no significant effects of chronic RS intake on the plasma C-max or AUC of daidzein or genistein or on the urinary C-max or AUC of either isoflavone (Table 3.4).

Table 3.4 Plasma and urinary C-max and AUC of daidzein and genistein after soy meals during chronic RS intake. Mean \pm SEM, range in parentheses, n = 6.

			soy meal			ANOVA [†]
			control	2 weeks RS	4 weeks RS	
Plasma	daidzein	C-max (ng/mL)	173 \pm 35.4 (85 – 315)	110 \pm 9.90 (70 – 124)	118 \pm 14 (67 – 155)	F _{2,4} = 1.311 p = 0.365
		AUC (ngmL ⁻¹ h)	946 \pm 145 (643 – 1611)	594 \pm 91 (223 – 830)	628 \pm 83 (350 – 909)	F _{2,4} = 3.224 p = 0.147
	genistein	C-max (ng/mL)	311 \pm 84.0 (164 – 710)	238 \pm 11.7 (214 – 288)	215 \pm 22.3 (143 – 276)	F _{2,4} = 1.933 p = 0.259
		AUC (ngmL ⁻¹ h)	1845 \pm 294 (1118 – 3159)	1405 \pm 71 (1133 – 1661)	1436 \pm 125 (1152 – 1836)	F _{2,4} = 1.845 p = 0.271
Urine	daidzein	C-max (μ g/mL)	8.2 \pm 4.7 (1.5 – 26.3)	11.4 \pm 4.4 (1.7 – 24.0)	11.7 \pm 3.7 (4.4 – 24.8)	F _{2,3} = 0.188 p = 0.837
		AUC (μ gmL ⁻¹ h)	32.2 \pm 17.0 (8.1 – 97.0)	37.2 \pm 12.4 (9.8 – 66.6)	38.0 \pm 9.0 (17.6 – 60.6)	F _{2,3} = 0.061 p = 0.942
	genistein	C-max (ng/mL)	7.7 \pm 5.3 (0.81 – 28.5)	5.1 \pm 1.5 (1.2 – 9.0)	6.4 \pm 1.7 (1.9 – 10.9)	F _{2,3} = 0.188 p = 0.837
		AUC (μ gmL ⁻¹ h)	28.5 \pm 19.1 (3.6 – 103)	19.7 \pm 6.5 (3.7 – 88.8)	23.1 \pm 5.8 (8.1 – 39.5)	F _{2,3} = 0.063 p = 0.940

[†]One-way ANOVA with repeated measures.

3.3.8 Effects of acute and chronic RS intake on plasma and urinary daidzein: genistein ratios

The ratio of daidzein: genistein in plasma was less than 0.6 and in urine was greater than 1.0 for all samples. Therefore, although the plasma level of daidzein was approximately half that of genistein, similar amounts were excreted in urine. There were no significant effects of either acute or chronic RS intake on these ratios (Table 3.5). However, there was a trend for the urinary daidzein: genistein ratio to have increased with 2 and 4 weeks RS intake, particularly overnight (11 – 24 h post-meal).

Table 3.5 Plasma and urinary daidzein: genistein ratios for acute and chronic RS study components. Mean \pm SEM.

			Sample time				11 - 24 h		ANOVA [†]			ANOVA [‡]
			5 h	7 h	9 h	11 h			F	df	p	
plasma	control soy	8	2.53 ± 0.33	2.27 ± 0.33	2.27 ± 0.42	2.19 ± 0.21	-	2.24 ± 0.39	0.776	3,4	0.565	F _{1,4} = 0.009 p = 0.929
	soy + RS	8	2.79 ± 0.31	2.13 ± 0.30	2.13 ± 0.33	2.15 ± 0.37	-	2.27 ± 0.27	2.116	3,3	0.277	
	control soy	6	2.37 ± 0.42	2.16 ± 0.40	2.16 ± 0.51	2.09 ± 0.23	-	2.09 ± 0.36	0.496	3,2	0.721	F _{2,1} = 15.700 p = 0.176
	2 weeks RS	6	2.51 ± 0.38	2.46 ± 0.33	2.68 ± 0.33	2.75 ± 0.31	-	2.74 ± 0.51	3.716	3,1	0.360	
	4 weeks RS	6	2.94 ± 0.51	2.38 ± 0.35	2.75 ± 0.69	2.12 ± 0.31	-	2.45 ± 0.32	2.374	3,2	0.310	
urine	control soy	7	0.84 ± 0.07	0.82 ± 0.12	0.77 ± 0.17	0.70 ± 0.24	1.11 ± 0.33	0.76 ± 0.12	0.227	3,4	0.874	F _{1,4} = 0.081 p = 0.789
	soy + RS	7	0.91 ± 0.1	0.77 ± 0.15	0.81 ± 0.18	0.55 ± 0.17	0.86 ± 0.23	0.76 ± 0.34	3.247	3,4	0.143	
	control soy	5	0.85 ± 0.08	0.72 ± 0.12	0.62 ± 0.11	0.68 ± 0.34	0.90 ± 0.34	0.67 ± 0.12	4.499	3,2	0.187	F _{2,3} = 0.235 p = 0.804
	2 weeks RS	5	0.89 ± 0.07	0.66 ± 0.15	0.60 ± 0.13	0.34 ± 0.10	0.29 ± 0.07	0.58 ± 0.09	7.449	3,2	0.121	
	4 weeks RS	5	0.94 ± 0.05	0.63 ± 0.13	0.64 ± 0.14	0.45 ± 0.17	0.53 ± 0.09	0.63 ± 0.12	1.118	3,2	0.504	

[†]One-way ANOVA with repeated measures for comparison of post-meal samples (5 – 11 h).

[‡]Two-way ANOVA with repeated measures for effect of acute or chronic RS across post-meal samples (5 – 11 h).

3.3.9 Effects of acute and chronic RS intake on daidzein metabolites, equol and ODMA

Plasma and urinary concentrations of the daidzein metabolites, equol and ODMA, were also determined. Three subjects had detectable levels of equol in multiple plasma samples and five subjects had equol in at least two urine samples (Table 3.6). Only one subject had equol in both plasma and urine samples and this was only following the soy meal after 4 weeks of RS intake. ODMA was not present in any plasma sample, but occurred somewhat randomly in the urine of four subjects after at least three of the four soy meals (Table 3.6).

Interestingly, although there had not been any notable differences between Kenyan and Caucasian subjects for daidzein or genistein bioavailability, the highest levels of urinary equol excreted throughout the study were by the two Kenyan subjects (subjects 6 and 9). Subject 6 had moderate levels of equol in their plasma after the soy meals at control and after 2 weeks RS but after 4 weeks RS, these levels were increased by an order of magnitude. Also after 4 weeks RS, this subject excreted the highest amount of urinary equol measured in the study. After the control meal, soy + RS and 2 weeks RS, the highest levels of urinary equol were excreted by subject 9, who unfortunately did not complete 4 weeks RS intake.

Table 3.6 Detection of equol and ODMA in plasma and urine samples during study. ns = no sample. All empty cells imply no equol or ODMA detected.

Subject	Plasma eql (ng/mL)				Urinary eql (µg/mL)					Urinary ODMA (µg/mL)				
	5h	7h	9h	11h	5h	7h	9h	11h	11-24h	5h	7h	9h	11h	11-24h
control soy														
1		38.1	88.4	84.8										
6				47.9										
2						0.5			0.6			0.8		0.8
3					0.3				ns	0.6				ns
8								0.3	0.3					0.6
9							0.8	1.6	1.6					
4											0.7	2.0		
soy + RS														
1		20.7	27.2	46.4										
2		49.6							0.4					1.1
9						0.5	0.4	0.3	4.8					
3									ns		0.6	0.4		ns
soy meal after 2 weeks RS														
1			34.3	32.7										
4	23.1	27.4	29.8	33.9					ns		0.6			ns
6	20.3	18.4												
8						0.1			0.2		1.6	0.7	0.2	0.2
9								1.3	3.8					
10						0.3	0.2							
2														1.2
3									ns			1.0		ns
soy meal after 4 weeks RS														
1			45.4	48.7										
4	27.7	24.9	30.2	30.5							0.7			
6	322	249	232	214	9.2	6.6	11	3.9	6.9					
8								0.9	0.6			1.4		0.6
2												1.9		0.9
3									ns		0.5			ns

When equol and ODMA were taken into account, there was a trend for the total [daidzein + equol + ODMA] in overnight urine samples to have increased after 2 and 4 weeks of RS intake compared with the control soy meal or soy + RS (Figure 3.11). However, as not all subjects completed overnight urine collections after all four soy meals, there were insufficient data to perform repeated measures ANOVA. There were no significant effects of the four test meals on overnight urinary [daidzein + equol + ODMA] concentration or total amount excreted ($F_{3,16} = 1.398$, $p = 0.280$ and $F_{3,16} = 1.566$, $p = 0.237$ respectively, one-way ANOVA). After 4 weeks RS intake, 2.9 ± 0.9 mg [daidzein + equol + ODMA] was excreted overnight, compared with 1.6 ± 0.5 mg daidzein.

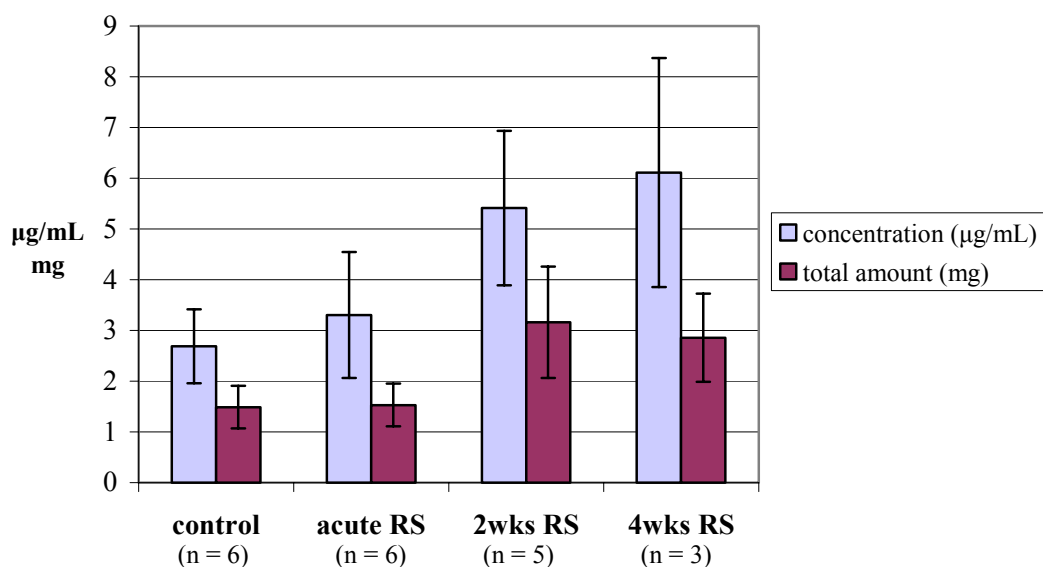


Figure 3.11 Mean overnight urinary excretion of daidzein + equol + ODMA. Excretion graphed as concentration in 11 – 24 h urine sample (µg/mL) and total amount excreted overnight (mg).

3.4 DISCUSSION

3.4.1 Isoflavone bioavailability after control soy meal

In this study, subjects consumed a 45 g serve of a soy-based cereal with 250 mL of soy milk, providing a total of approximately 65 mg isoflavones. This is the equivalent of two serves of soy-containing foods, similar to the daily exposure in a consumer of a vegetarian-based diet and is representative of the upper levels of habitual daily consumption in many Asian populations. Based on the average mass of subjects, our daily dose was $1.05 \pm$ mg total isoflavones per kg body weight, and as such, is in line with dietary intakes of people consuming soy as a staple food, up to 0.4 mg daidzein/kg body weight and 0.75 mg genistein/kg body weight (Setchell *et al.*, 2003b).

After intake of the soy meal, plasma concentrations of both daidzein and genistein were significantly increased in all post-meal samples compared with baseline (Figure 3.2). There was large variability in the response of subjects in their time to maximum concentration and between the shapes of the absorption curves for individual subjects. On average, the peak plasma isoflavone levels occurred 7 hours post-meal for both daidzein and genistein, with concentrations of 126 ng/mL (0.5 μ mol/L) and 285 ng/mL (1.06 μ mol/L), respectively. These are within the reported range of 50-800 ng/mL total isoflavones occurring in plasma in adults consuming modest quantities of soy foods containing about 50 mg/day total isoflavones (Setchell and Cassidy, 1999).

Other studies have also reported maximum plasma isoflavone concentrations between 7 and 9 hours after soy intake (King and Bursill, 1998; Setchell and Cassidy, 1999; Setchell *et al.*, 2003b) and the maximum plasma levels in this study are similar to those reported in other pharmacokinetic studies that have determined plasma isoflavone levels after soy food intake. In the current study, 250 mL soy milk provided approximately 1/3 of the total isoflavones in the soy meal and the maximum plasma daidzein and genistein concentrations were approximately 3 times greater than those reported by Setchell and

others (2002) after ingestion of 250 mL soy milk. Other studies (King and Bursill, 1998; Xu *et al.*, 1994) used higher isoflavone doses in liquid matrices and the maximum plasma concentrations were higher still. However, more rapid elimination is observed for isoflavones in a liquid matrix (Setchell, 2000), which may affect isoflavone bioavailability. At 11 hours post-meal in the current study, the plasma isoflavone levels were still elevated from baseline and not statistically different to those at 5 hours, suggesting that plasma clearance of the isoflavones does not occur until at least 11 hours after their consumption. Watanabe and others (1998) observed a second shoulder or peak at 12 hours in the plasma of some subjects after a soy intake, suggesting that these isoflavones continue to be absorbed for at least 12 hours after intake and King and Bursill, (1998) reported that plasma total isoflavone concentrations remained above 50% of their maximum values for about 12 - 16 hours. Isoflavone levels generally return to baseline by about 48 hours after soy intake (Watanabe *et al.*, 1998).

In the current study, the plasma concentration of genistein was approximately double that of daidzein for all samples after soy intake, consistent with the findings of other similar studies (King and Bursill, 1998; Setchell *et al.*, 2003b). Since genistein and its glycosides tend to be present at higher levels than daidzein and its glycosides in most foods, it is to be expected that higher plasma genistein is reported after soy intake (King and Bursill, 1998; Setchell *et al.*, 2001). However, higher serum concentrations of genistein occur independent of the composition of the food, even when intake levels of daidzein and genistein are equivalent (Setchell *et al.*, 2003b). In contrast, urinary concentrations of daidzein and genistein were similar. This may reflect different pharmacokinetic regimes of absorption, excretion or metabolism. Most other studies also report greater urinary excretion of daidzein compared with genistein, which has been explained based on structural differences between these two compounds. It has been suggested that the lower molecular weight and greater water solubility of daidzein could account for its higher urinary excretion (Xu *et al.*, 1994), whereas the less hydrophilic nature of genistein may promote its excretion in bile (King and Bursill, 1998). Low recoveries of the isoflavones in urine indicate significant bacterial degradation and possible metabolism to other unidentified compounds (Lampe *et al.*, 1998; Xu *et al.*,

1994), which would also contribute to variation between individuals. Daidzein and genistein followed a similar pattern of excretion, both with maximum excretion 7 hours post-meal; others have also reported parallel excretion profiles of daidzein and genistein with maximum urinary isoflavone excretion 7 hours after soy intake (Lu *et al.*, 1995; Watanabe *et al.*, 1998).

3.4.2 Effects of acute RS intake on isoflavone bioavailability

The addition of RS to the soy meal significantly reduced plasma daidzein and genistein levels between 5 and 11 hours post-meal, suggesting that acute RS impeded plasma absorption and bioavailability of these isoflavones (Figure 3.5). Urinary excretion of daidzein and genistein also tended to be reduced, particularly 7 hours post-meal which was when the peak mean excretion occurred after the control soy meal; however, this was not significant (Figure 3.6). These similar reductions in both plasma and urinary isoflavones suggest that less isoflavone was initially absorbed and correspondingly there was reduced excretion. The peak mean plasma concentrations occurred later for both daidzein and genistein after the soy + RS meal compared with the control soy meal (9 hours compared with 7 hours) as did the peak mean urinary excretion of daidzein (11 hours post-meal compared with 7 hours) which may suggest a delay in isoflavone absorption with acute RS intake. The constant excretion rate of 2 µg/mL of genistein over the 24 hour sampling period after soy + RS also suggests a slowed absorption and steady excretion.

Although there was a trend for the mean plasma daidzein and genistein concentrations to decline between 9 and 11 hours after the soy + RS meal, indicating that the maximum concentrations had been reached prior to this time, others have reported a second peak in plasma levels due to enterohepatic recycling (Watanabe *et al.*, 1998). In this study, a second peak may have been evident after the last plasma sample 11 hours post-meal. King and Bursill, (1998) reported that total plasma concentrations remained above 50% of their maximum values for about 12-16 hours after a soy intake, so if the original

absorption was delayed rather than reduced, plasma levels of isoflavones may still have been elevated for up to 24 hours after the soy + RS meal. In addition, acute RS intake did not significantly reduce the post-meal urinary excretion; nor did it affect the overnight excretion in terms of both rate and total amount of each isoflavone, implying that there was no overall reduction in isoflavone bioavailability over the 24 hours post-meal. However, large inter-individual variation may have masked the significance of the reduction in urinary isoflavone excretion in the first 11 hours post-meal.

A number of factors can influence the absorption of food components, including dietary habits, the food matrix, extent of intestinal fermentation and intestinal transit time (Zubik and Meydani, 2003). Although resistant starch can have prebiotic effects in the large intestine, at least two weeks of regular intake are necessary for related bacterial changes (Topping and Clifton, 2001); thus the acute effects of resistant starch intake are not a result of its prebiotic capacity. Rather, it is more likely that other effects of resistant starch, including its adsorption of other dietary constituents, increases in intestinal contents and faecal output or a reduction in intestinal transit time (Brown *et al.*, 2000) were the likely cause of the decrease and/or delay in absorption of isoflavones from soy reported here.

Resistant starch is capable of adsorbing or binding certain compounds from foods (Brown *et al.*, 1999). In the current study, it is possible that the ingested RS effectively bound a proportion of the isoflavones, either inhibiting or slowing their absorption from the gastrointestinal tract. This would explain both the inhibition of plasma absorption 5 – 11 hours post-meal and the delay in urinary excretion over this time. Although there is no information on the estrogen or phytoestrogen-binding ability of RS, dietary fibres from a variety of sources have been shown to bind with conjugated and unconjugated estrogens *in vitro* (Shultz and Howie, 1986). The β -glucosidase enzymes in the small intestine are mostly membrane bound (Day *et al.*, 1998) and as they effectively facilitate uptake of the aglycones after hydrolysis, it therefore seems more likely that the RS has bound the glycosidic forms of the isoflavones prior to their initial hydrolysis rather than the aglycones.

Resistant starch increases intestinal content and faecal bulk (Brown *et al.*, 2000), which can result in dilution of bacterial numbers and decreases in intestinal β -glucuronidase and β -glucosidase activities (Hylla *et al.*, 1998; Tew *et al.*, 1996; Zhang *et al.*, 1999). The addition of 20 g RS to the soy meal would have been sufficient to produce these effects and may explain the decreased plasma and urinary isoflavones. Increased intestinal and faecal bulk are positively correlated with faecal excretion of estrogens, and negatively correlated with plasma and urinary estrogens (Adlercreutz *et al.*, 1987). Further, dietary components such as fibre can specifically affect the enterohepatic circulation of certain compounds including estrogens and likely, isoflavones (Lampe *et al.*, 1994). Similarly, acute RS intake may have affected bacterial enzymatic activity and enterohepatic circulation and consequently, hydrolysis of isoflavone glycosides and absorption of aglycones. Ingested conjugated isoflavones require initial hydrolysis for absorption; further reabsorption of estrogens and phytoestrogens during enterohepatic recirculation requires hydrolysis by β -glucuronidases. The decreases in plasma isoflavones in the 11 hours post-meal in the current study may also be indicative of slowed enterohepatic recirculation.

Because intestinal transit time is decreased with RS consumption, acute resistant starch intake may have decreased the time available for isoflavone hydrolysis and absorption. However, an inherently shorter gut transit time may increase the bioavailability of the secondary metabolites of isoflavones due to less opportunity for degradation before arrival in the colon (Hendrich, 2002; Zheng *et al.*, 2003). The additional effects of dietary components on this characteristic have not been examined and there may be a critical exposure time necessary for optimal absorption, and transit times shorter than this may reduce absorption.

Whilst the initial glycoside hydrolysis has been suggested to be the rate limiting step in isoflavone absorption and metabolism (Izumi *et al.*, 2000; Setchell *et al.*, 2001; Steer *et al.*, 2003) and thus essential for isoflavone bioavailability, ingestion of the glucoside conjugates rather than aglycones, results in greater isoflavone bioavailability, due to longer absorption and excretion kinetics (Setchell *et al.*, 2002b; Zubik and Meydani,

2003). So, although acute RS intake may reduce or delay the initial hydrolysis of isoflavones, either through a reduction in this activity or binding of the isoflavones, this may facilitate increased bioavailability analogous to that of isoflavone glucoside ingestion.

In a similar study, the addition of approximately 25 g insoluble fibre from wheat bran to a single soy meal significantly reduced (50%) plasma genistein and daidzein levels 24 hours after intake and decreased recovery of total isoflavones compared with a control soy meal (Tew *et al.*, 1996). This was attributed to the binding of isoflavones to the insoluble fibre. The larger reduction in plasma isoflavones 24 hours post-meal and the decreased urinary recovery in the wheat fibre study may be due to the greater estrogen-binding ability of wheat bran compared with corn, from which RS is derived, demonstrated *in vitro* (Rose *et al.*, 1991). This may translate to a similar effect *in vivo* with isoflavones. In addition, wheat bran is a more effective bulking agent (Topping and Clifton, 2001) and causes a greater reduction in transit time than RS (Ferguson *et al.*, 2000).

Overall, the consumption of resistant starch in the same meal as soy significantly reduced plasma daidzein and genistein concentrations compared with after the soy meal alone. There were also trends for decreased urinary isoflavone excretion between 5 and 11 h post-meal, of which large inter-individual variability may have masked statistical significance and for delayed peak plasma isoflavone absorption and daidzein excretion. It is likely that the RS bound the isoflavones, thereby reducing their absorption, although it is also possible that isoflavone absorption was delayed, rather than reduced. However, to distinguish between these effects, an increased plasma and urinary sampling time as well as faecal analysis of isoflavones would be needed.

3.4.3 Effect of chronic RS intake on isoflavone bioavailability

Chronic RS consumption decreased plasma daidzein and genistein levels compared with the control soy meal with some significant effects. In contrast, urinary isoflavone excretion was not reduced between 5 and 11 hours post-meal and the total amount of daidzein excreted overnight after 2 weeks RS, increased for all subjects, although not significantly. There were mean percent increases in the urinary excretion of both daidzein and genistein after 2 and 4 weeks RS intake for all post-meal samples and AUC, although again these were not significant, possibly due to the large inter-individual variation. The trend for mean increases in urinary isoflavones implies that the bioavailability of the isoflavones was enhanced with chronic RS intake. Although the plasma absorption was decreased in the 5 – 11 hour post-meal time period, the increases in urinary excretion 11 – 24 hours after the meal suggest that absorption was delayed and hence peak plasma levels may not have been reached until after 11 hours. However, as there were still increases in urinary isoflavones, particularly daidzein between 5 and 7 hours, this may indicate prolonged enterohepatic circulation or a slowed release in the plasma or more partitioning into tissue rather than a delay in the initial absorption. Overall, the effects of chronic RS intake on plasma and urine were not different after 4 weeks RS intake compared with 2 weeks, except for the increased overnight daidzein excretion at 2 weeks. However, the decreased daidzein excretion at 4 weeks compared with 2 weeks RS intake was caused by one subject who after 4 weeks of RS intake, had reduced urinary daidzein but increased equol excretion.

As RS was not consumed with the soy meals during chronic RS intake, any changes in isoflavone bioavailability observed are likely to be due to the prebiotic effects of RS rather than any acute effects. RS escapes digestion in the upper intestinal tract (Gibson, 1998; Yue and Waring, 1995) and can act as a prebiotic in the large intestine, where it is fermented by the resident microflora and stimulates bacterial growth, especially of bifidobacteria (Brown *et al.*, 1999). Bifidobacteria and bacteroides comprise the majority of microorganisms in the human gastrointestinal tract (Friend and Chang, 1984), and these, along with lactobacilli, are the most important producers of β -glucosidase

activity (Steer *et al.*, 2003; Uehara *et al.*, 2001; Xu *et al.*, 1995). *In vitro*, bifidobacteria can hydrolyse daidzin and genistin from soy milk to their respective aglycones and further metabolise daidzein to equol (Tsangalis *et al.*, 2002), and in human faecal samples can also metabolise daidzein (Chang and Nair, 1995; Kim *et al.*, 1998). In rats, intestinal and faecal β -glucosidase activity increases in response to increases in bifido- and other bacteria from RS feeding (Silvi *et al.*, 1999; Wang *et al.*, 2002b) and the metabolic capacities of bifidobacteria strains are correlated with β -glucosidase activity (Tsangalis *et al.*, 2002). Thus, the effects reported here for chronic RS treatment on isoflavone bioavailability may be mediated through an increase in bifidobacteria and its β -glucosidase activity. In addition, RS consumption also decreases faecal pH (Hylla *et al.*, 1998; Phillips *et al.*, 1995), promoting growth of lactic acid bacteria (Boever *et al.*, 2000) and further enhancing β -glucosidase activity (Steer *et al.*, 2003), and potentially isoflavone metabolism.

Although there are no studies to date that have specifically tested the effects of RS intake on isoflavone bioavailability, those of a similar prebiotic, fructooligosaccharide (FOS) have been examined. Seven days intake of 10 g FOS per day significantly increased faecal bifidobacteria in humans (Bouhnik *et al.*, 1999). However, FOS is fermented rapidly and completely; whereas RS has a slow, prolonged fermentation of only about 70 – 80% (Alles *et al.*, 1997). Nevertheless, Uehara and colleagues (2001) demonstrated that in rats, seven days of a 5% FOS diet increased the bioavailability of a single intragastric administration of isoflavone conjugates. Plasma bioavailability of both daidzein and genistein and the urinary excretion of genistein were all significantly increased 24 – 48 hours after administration and there was also an increase in time to peak portal vein concentration of daidzein. These researchers concluded that FOS enhanced the enterohepatic recirculation and/or large intestinal absorption of genistein, and prolonged the clearance of both isoflavones. The significant increase in urinary isoflavones after 24 hours is important, as this also implies there is a delay in either absorption or excretion. A longer sampling period in our study may have revealed greater effects of RS.

In an *in vitro* system examining the metabolism of genistin to genistein, addition of FOS increased bifidobacteria and lactobacilli populations as well as the concentration of genistein (Steer *et al.*, 2003). However, these authors were not able to determine whether this enhanced conversion of genistin to genistein was a result of increases in bifidobacteria and lactobacilli or of decreases in other bacteria which are responsible for the further degradation of genistein to inactive products. Similarly, if RS can reduce the proportions of bacteria responsible for the degradation of isoflavones to inactive products, isoflavone bioavailability may be enhanced (Xu *et al.*, 1995).

Overall, there were some significant reductions in plasma isoflavone levels with chronic RS intake and trends towards increased daidzein excretion. These effects may indicate delayed isoflavone absorption and excretion which are likely due to prebiotic effects of RS on gut microflora and consequently, effects on enzyme activity, enterohepatic circulation and isoflavone bioavailability. Further investigation of this result, with a more comprehensive plasma and urine sampling regime and faecal sample analysis would help in the elucidation of the mechanisms of this effect on isoflavone bioavailability.

3.4.4 Effects of chronic RS intake on daidzein metabolites

Although all subjects were able to absorb and excrete daidzein and genistein, only three subjects had equol in their plasma and four excreted urinary equol; these were not always the same subjects. Chronic RS intake increased equol absorption and/or excretion in some subjects. One subject (who unfortunately did not provide urine samples) had equol in their plasma after all four test meals; acute RS intake decreased and chronic RS intake delayed their equol absorption. For two other subjects, equol was only present in their plasma during the chronic RS phase and for three subjects, chronic RS intake increased urinary equol excretion. This suggests that equol excretion and bioavailability were increased after chronic RS intake.

Urinary excretion of equol and ODMA in the Caucasian subjects in this study was similar to the 24 h levels reported by Lampe and colleagues (2001), of approximately 0.5 mg and 0.6 mg, respectively after 3 days of soy protein intake. In contrast, the Kenyan subjects excreted between 1 and 3 mg equol in the overnight samples. Rowland and co-authors (1999b) reported two orders of magnitude difference between equol excretion of individuals after consumption of soy protein flour, of up to approximately 2 – 3 mg equol per day. After intake of 40 g of soybean for five days, subjects excreted approximately 1 mg equol during the first 24 hours after ingestion, with maximum excretion of approximately 4 – 5 mg/day after 3 days (Axelson and Setchell, 1980). Although the detection of equol in plasma and urine in the current study may thus have been limited by the restricted sampling protocol, as there were no plasma samples collected later than 11 hours post-meal and no urine samples later than 24 hours post-meal, the sporadic occurrence in plasma and urine may highlight the varied capacities of individuals in their ability to metabolise daidzein to equol. Furthermore, ODMA was not detected in the urine of the two Kenyan subjects who produced equol, but was consistently excreted by another subject had no equol in plasma and only minimal amounts in urine. Although correlations could not be conducted due to the inconsistent presence of equol and ODMA in plasma and urine, these findings may suggest an inverse relation between metabolism of daidzein to equol and ODMA, as was also observed by Kelly and colleagues (1995).

Although no other studies have yet specifically examined a prebiotic role in the metabolism of daidzein to equol in humans, the essential role of gut microflora in daidzein metabolism is well established and has been noted (Adlercreutz, 1995, 1998; Lampe *et al.*, 1998; Lampe *et al.*, 2001; Rowland, 1999; Rowland *et al.*, 2000). In mice, addition of 5% FOS to a diet containing isoflavones increased both cecal β -glucosidase activity and equol production compared with isoflavones alone, without any changes in plasma daidzein or genistein (Ohta *et al.*, 2002). However, rodents are constitutive equol-producers in contrast to humans (King, 1998), which may be related to differences in metabolism and thus results may not be able to be extrapolated. Further, soy is a common component of animal feed and may promote certain gut microflora across generations that may enhance equol production. Uehara and colleagues (2001) suggested

that no increase in urinary daidzein after a single isoflavone administration in rats fed a 5% FOS diet, may have been due to an enhancement in metabolism to other compounds, but these were not measured.

In premenopausal women known to be either equol-excretors (n = 10) or non-excretors (n = 9), a 1 month intervention of wheat bran cereal containing 16 g dietary fibre had no effect on urinary equol levels after soy intake (Lampe *et al.*, 2001). Even though this level of wheat bran fibre would have been sufficient to alter the colonic environment in terms of transit time and faecal bulk, it lacks a prebiotic effect on the gut microflora. This suggests that it is the specific prebiotic capacity of RS that may be responsible for the enhanced conversion of daidzein to equol. More than one bacterial species appears to be involved in this metabolic conversion (Hur *et al.*, 2000) and strains of bifidobacteria (Kim *et al.*, 1998), streptococci, ruminococci and bacteroides (Ueno and Uchiyama, 2001) and *Escherichia coli* (Hur *et al.*, 2000) have been identified as producing this metabolic activity. In rats, RS also increases lactobacilli, streptococci and enterobacteria (Silvi *et al.*, 1999; Wang *et al.*, 2002b).

3.4.5 Influence of diet on equol production

While it is consistently reported that only about 30 – 40% of individuals from various populations excrete equol after consuming soy products (Lampe *et al.*, 1998; Lampe *et al.*, 2001; Slavin *et al.*, 1998), the factors related to the metabolism of daidzein to equol remain unknown. The clear distinction between individuals in their capacity or inability to produce equol (Rowland, 1999) may be an inherent characteristic related to genetics or habitual diet which is not modifiable by dietary interventions (Karr *et al.*, 1997; Lampe *et al.*, 2001). However, the unequivocal role of the gut microflora in this conversion may allow for potential modification of this activity through dietary components including prebiotic treatment. In particular, the type and amount of dietary carbohydrate available *in vivo* to intestinal microflora may be important for equol-producing capacity (Lampe *et al.*, 2001; Rowland, 1999). In an *in vitro* colonic fermentation system, it was found that

a high carbohydrate environment increased fermentation and the rate of conversion of daidzein to equol (Setchell and Cassidy, 1999). Amongst other dietary components, carbohydrate (Lampe *et al.*, 2001), non-starch polysaccharides (Rowland, 1999) and soluble and insoluble dietary fibre (Lampe *et al.*, 1998) have all been positively associated with an individual's ability to produce equol.

3.4.6 Equol production in Kenyan subjects

The results of this study suggest a potential role of RS in increasing daidzein metabolism to equol via prebiotic activities; however, not all subjects appear to be predisposed to this effect. The high occurrence of, and specific increases in, plasma and/or urinary equol in the two Kenyan subjects, suggests an influence of genetics, or early dietary exposure and gut microflora development on the ability to produce equol. It should be noted that although the two Kenyan subjects had been living in Australia for approximately one year prior to the study, they still consumed Kenyan-type meals and tofu and soy milk regularly. However, despite the current diets of the Kenyans being mostly comparable with those of the Australian subjects, dissimilar previous habitual diets may have resulted in development of different gut microfloral profiles. The increased prevalence of urinary equol in the two Kenyan subjects in the present study may be related to the high carbohydrate and resistant starch content of their traditional diet. The traditional Kenyan diet is high in carbohydrates, dietary fibre and resistant starch from unrefined whole grain cereals, legumes and other plant foods of limited digestibility (Mukeshi and Thairu, 1993; Oniang'o and Komokoti, 1999; Topping and Clifton, 2001). In a review of Kenyans aged between 19 and 31 years, it was found that the average daily carbohydrate intake was 75% of total energy (between 330 and 440 g/day), much higher than intakes recommended in developed Western countries (Mukeshi and Thairu, 1993). Maize, from which RS is derived, is the predominant ingredient in most of the commonly consumed dishes in Kenya, including ugali, a national dish prepared from maize flour (Oniang'o and Komokoti, 1999); githeri, maize and kidney beans; sukumawiki, maize flour and kale; and porridge (Mukeshi and Thairu, 1993). Maize porridge is often consumed

reheated or cold, which has greater RS content (Ahmed *et al.*, 2000; Annison and Topping, 1994), classified as retrograded RS (Brown *et al.*, 1995). In fact, the term “resistant starch” was originally used to describe the incomplete *in vitro* digestion of foods that had been cooked and then cooled (Berry, 1986). The contribution of legume products in the Kenyan diet may also be important, as a recent evaluation of starch foods found these contained the highest amounts of resistant starch, 10 and 11% RS (total starch basis) for brown and white beans respectively (Elmstahl, 2002). Therefore, early dietary exposure to high intakes of resistant starch may predispose individuals to particular metabolic capacities, such as the production of equol from daidzein, or to specific gut microflora responses to dietary treatments.

3.4.7 Influence of background diet and genetics on gut microflora

Background diet and inherent characteristics are expected to affect the gut microflora population, and hence an individual’s capability for equol production (Lampe *et al.*, 1998), and thus it is unlikely that a novel diet will have greater impact than the habitual diet in relation to equol-excretor status (Lampe *et al.*, 2001). Gut microflora has been shown to differ between populations (Thadepalli *et al.*, 1979) and the relative contributions of background diet or genetics to this have not been established. In one cultural comparison, Canadians had a higher percentage of total bacteria as *Lactobacillus* and *Clostridium* species and a lower percentage as *Bifidobacterium* and *Eubacterium* species, while the latter two were the dominating bacterial species in the gastrointestinal tract of rural Japanese (Lampe *et al.*, 1998). The Japanese also had a higher intake of soy food and were consuming approximately 60% of daily energy intake from carbohydrate (Lampe *et al.*, 1998). Interestingly, eubacteria are known to increase in subjects after chronic daily RS consumption (Schwartz *et al.*, 2002).

Hendrich and co-authors (1998) and Zheng and colleagues (2003) have identified distinct and significantly different isoflavone disappearance phenotypes for both daidzein and genistein from human faecal samples, with differences in the distribution of phenotypes

between Chinese and Caucasian women (Zheng *et al.*, 2003). Faecal degradation is directly related to, and thus an important indicator of, gut microfloral activity and is also a determinant of plasma and urinary isoflavone levels (Zhang *et al.*, 1999). There is evidence that isoflavone metabolism is a consistent feature of individuals, as both the degradation phenotypes (Hendrich *et al.*, 1998) and urinary isoflavone excretion patterns have been shown to be stable after repeated testing in subjects (Kelly *et al.*, 1993).

In addition, gastrointestinal transit time is reported to be faster in Chinese than Caucasian subjects, resulting in increased isoflavone bioavailability in the Chinese subjects (Zheng *et al.*, 2003). A longer gastrointestinal transit time may decrease isoflavone bioavailability as this would provide more opportunity for isoflavones to be degraded, resulting in less available for absorption (Hendrich, 2002). In addition, the effect of RS will also be dependent on an individual's inherent characteristics, with rapid transit of the small intestine likely to deliver more starch into the large bowel (Topping and Clifton, 2001), where it will be acted upon by the colonic bacterial population and have greater likelihood of producing a prebiotic effect. The overall effect of transit time on isoflavone bioavailability has not been established and appears to be a balance between sufficient time for absorption and excess time to allow degradation.

3.4.8 Individual variability and dietary intakes

A consistent finding in most isoflavone bioavailability studies using human subjects is the high inter-individual variation. To avoid three of the many potential confounding factors related to this variation, we chose subjects of a single gender (female), comparable hormone status (pre-menopausal), and with similar background diets (non-vegetarian - omnivorous). However, two of the subjects were Kenyan, and although they have lived in Australia for the year prior to the study, they had retained some traditional Kenyan dietary habits including regular consumption of soy products such as tofu and soy milk. In addition, during development, the gut microflora of these subjects would have been exposed to different foods compared with that of the Australian subjects.

In this study, all subjects consumed the same isoflavone dose in the test soy meals, however other studies have delivered isoflavone doses based on body weight (King and Bursill, 1998; Xu *et al.*, 1994; Xu *et al.*, 1995). The average weight of the 9 subjects in the current study was 62 ± 3 kg, with a range of 48 – 74 kg. This absolute difference of 26 kg is a large proportion (almost 50%) of the smallest body weight, so may be a cause for some of the variation. However, there were no trends for plasma or urinary isoflavone levels related to body mass. When Zubik and Meydani (2003) adjusted plasma isoflavone concentrations by BMI in their study, this did not change any of the results; however adjustment for total mass may have been more appropriate.

Some of the variability in the effects of RS intake may be explained by the large individual variation that is known to occur in response to fermentation of prebiotics (Cummings *et al.*, 2001). The amount of resistant starch reaching the large intestine for prebiotic action after intake may vary between subjects, as only incompletely digested starch can contribute to this component and small intestinal amylolysis can occur at different rates (Topping and Clifton, 2001). Intestinal transit time may also have an effect as a rapid small intestinal transit time results in less complete fermentation (Baghurst *et al.*, 1996) and thus delivery of more starch into the large intestine (Topping and Clifton 2001). However, Cummings and co-authors (1996) suggested that the presence or absence of RS-degrading bacteria in the gut was the largest determinant of variable abilities of individuals to degrade RS in the large intestine. Individual differences in the contribution of gut flora to RS utilisation will also affect the complementary contribution of RS to the composition of the flora (Cummings *et al.*, 1996). Therefore an individual's gut microflora can potentially influence resistant starch fermentation in addition to equol production.

In the chronic component of this study, a daily intake of 20 g RS was incorporated into the usual diets of subjects and was generally well tolerated. This daily intake is the amount reported to be required for positive physiological benefits (Baghurst *et al.*, 1996; Brown *et al.*, 2000) and greatly exceeds the typical consumption of RS in Australians of about 5 - 7 g/day (Olesen and Gudmand-Hoyer, 1997). While most subjects reported

increased flatulence and bloating, these are common side-effects of daily prebiotic consumption (Cummings *et al.*, 2001) and only one subject withdrew from the study due to discomfort caused by these symptoms. Although we did not measure any markers for alteration in gastrointestinal function, 20 g of daily RS intake for 2 and 4 weeks has previously been shown to be sufficient to induce gastrointestinal changes and bacterial adaptations (Bouhnik *et al.*, 1999; Cummings *et al.*, 2001; Phillips *et al.*, 1995; Schwartz *et al.*, 2002; Topping and Clifton, 2001).

3.4.9 Conclusions and recommendations

The results of this pilot study suggest that when included in the same meal as soy, RS can reduce or delay the plasma absorption of isoflavones. However, chronic RS consumption may enhance isoflavone bioavailability as suggested by increased urinary excretion of daidzein and genistein, and increase the metabolism of daidzein to equol in some subjects. These effects may be mediated by the prebiotic effects of RS on gut microflora. The increased prevalence of equol in two Kenyan subjects may indicate that pathways of isoflavone metabolism and their amenability to dietary modifications, may be related to genetics or lifetime dietary exposure-related differences in gut microflora. Furthermore, gut microflora may in the first instance determine an individual's response to prebiotic intake.

This potential for chronic RS intake to increase isoflavone bioavailability, particularly that of equol, is an interesting finding and has not been reported previously. However, a more comprehensive sampling regime in a larger study population would be needed to confirm these effects and to enable more thorough investigation. In addition, further investigation of the finding reported here of increased equol production with RS intake in Kenyan subjects may assist in understanding some of the determinants of this process. The results of this pilot study formed the basis for the hypotheses in the study reported in Chapter 5, that chronic dietary intake of soy, in combination with either RS or a probiotic, may increase isoflavone bioavailability.

3.5 APPENDIX

Appendix 3.1 Mean plasma (ng/mL) and urinary (µg/mL) isoflavone concentrations following soy meals (control and 2 week RS) for all subjects and only those who completed 4 weeks RS intake. pl = plasma, ur = urine. O/N = overnight.

O/N (mg)								ANOVA [†]	
control	n	5 h	7 h	9 h	11 h	11-24 h			
daidzein	pl	9	106 ± 27	125 ± 17	120 ± 16	107 ± 18		F = 0.328	
		6	114 ± 33	133 ± 20	97 ± 13	86 ± 9		p = 0.578	
	ur	9	3.0 ± 0.9	7.8 ± 3.0	6.3 ± 2.2	4.5 ± 1.8	2.0 ± 0.7	1.1 ± 0.3	F = 0.009
		5	2.9 ± 1.3	7.3 ± 4.8	6.0 ± 3.5	2.8 ± 1.5	2.3 ± 1.0	1.1 ± 0.4	p = 0.927
genistein	pl	9	222 ± 24	278 ± 62	247 ± 45	247 ± 57			F = 0.317
		6	222 ± 25	285 ± 66	259 ± 46	258 ± 60			p = 0.584
	ur	9	2.5 ± 0.7	7.6 ± 3.4	5.3 ± 2.3	3.3 ± 1.3	2.2 ± 1.2	0.9 ± 0.3	F = 0.000
		5	2.5 ± 1.1	7.0 ± 5.4	5.1 ± 3.7	1.8 ± 0.9	2.5 ± 1.8	0.8 ± 0.3	p = 0.984
2 weeks RS									
daidzein	pl	9	71 ± 17	100 ± 12	99 ± 15	68 ± 13			F = 0.495
		6	65 ± 22	89 ± 12	71 ± 13	50 ± 11			p = 0.500
	ur	9	4.6 ± 1.3	7.0 ± 2.6	7.4 ± 2.5	6.5 ± 2.2	4.3 ± 1.5	2.6 ± 1.1	F = 0.007
		5	4.0 ± 1.5	7.6 ± 4.2	6.8 ± 3.4	4.5 ± 2.4	5.5 ± 2.5	3.4 ± 1.8	p = 0.935
genistein	pl	9	160 ± 18	223 ± 27	218 ± 32	165 ± 33			F = 0.562
		6	154 ± 21	203 ± 24	176 ± 25	129 ± 24			P = 0.471
	ur	9	3.8 ± 0.9	4.7 ± 1.1	5.3 ± 2.0	4.8 ± 2.1	2.1 ± 0.9	1.1 ± 0.5	F = 0.681
		5	3.3 ± 1.1	3.4 ± 1.1	3.8 ± 1.7	2.1 ± 1.2	1.3 ± 0.6	0.7 ± 0.3	P = 0.441

[†] Two-way ANOVA with repeated measures and between groups analysis for samples 5 – 11 h.

CHAPTER 4

EFFECTS OF SOY VS. DAIRY PRODUCTS ON ISOFLAVONE BIOAVAILABILITY AND LIPIDS IN MILDLY HYPERLIPIDEMIC SUBJECTS : A 10 WEEK CROSSOVER STUDY

Study Context and Collaborations

This dietary intervention was conducted by a team of researchers in the Smart Foods Centre at the University of Wollongong in partnership with the industry sponsor, So Natural Foods (Taren Point, NSW, Australia) and has been published (BJ Meyer, TA Larkin, AJ Owen, LB Astheimer, LC Tapsell, PRC Howe (2004) *Limited Lipid-Lowering Effects of Regular Consumption of Whole Soybean Foods*. Annals of Nutrition and Metabolism 48: 67-78).

As a team member, my role was to contribute to the design, execution and interpretation of the study and to analyse the isoflavone content of plasma and urine. This chapter contains the results of my isoflavone analysis as well as analysis of plasma lipids to provide context, though I did not conduct the laboratory analysis for the latter. However, the data analyses, the tables and graphs included and further analyses of factors affecting isoflavone bioavailability and lipid effects are all my own work as are the points made in discussion.

4.1 INTRODUCTION

In 1995, a meta-analysis of 38 clinical trials found a significant reduction in total cholesterol, LDL-cholesterol and triglycerides with soy protein intake (Anderson *et al.*, 1995) and in 1999 the U.S. Food and Drug Administration authorized a health claim for soy protein. However, there is still discrepancy in the hypocholesterolemic effect of soy and the relative contributions of soy protein and isoflavones. As introduced in Chapter 1, it appears that the combination of both soy protein and the isoflavones are necessary for a lipid-lowering effect, suggesting a synergistic action (Potter, 1998).

While most studies examining the effects of soy on lipids have used soy protein isolates or extracts containing both soy protein and isoflavones (Crouse *et al.*, 1999; Steinberg *et al.*, 2003; Wong *et al.*, 1998), foods made from whole soy beans may provide more health beneficial effects as they contain both soy protein and isoflavones intact in their natural matrix. Whole soy bean extracts also contain the n-3 fatty acid α -linolenic acid, which itself has cardioprotective effects (Holub, 2002; Wijendran and Hayes, 2004). In addition, the use of soy protein isolate in soy milk production results in a lower isoflavone content than soy milk made from whole soy beans, due to losses of approximately 50% during extraction (Coward *et al.*, 1993; Tsangalis *et al.*, 2002; Wang and Murphy, 1996).

This study used soy milk and soy yoghurt prepared from whole soy bean extracts in a dietary intervention to determine isoflavone bioavailability and lipid effects. Mildly hyperlipidemic subjects followed two dietary treatments of 5 weeks each in a crossover design to compare the effects of soy intake with a dairy control on isoflavone bioavailability and lipids.

4.2 METHODS

4.2.1 Subjects

Men and postmenopausal women older than 50 years were recruited through the local media. Selection criteria included total plasma cholesterol greater than 5.5 mmol/L and/or mildly elevated blood pressure (>140/90mm Hg) and it was essential that subjects were not taking medication for either condition. Subjects were chosen who did not habitually consume soy-based foods and whose diets were not high in n-3 fatty acids. Twenty six subjects (16 males and 10 females) began the intervention. This study was approved by the Human Research Ethics Committee at the University of Wollongong (HE 00/157).

4.2.2 Study design

The study had a randomised crossover design with two 5-week dietary periods, not separated by a washout period (Figure 4.1). Initially, there was a 2 week run-in phase during which subjects were required to incorporate 4 serves of low fat dairy milk and/or yoghurt into their normal diets. This enabled assessment of whether subjects could comply with this dietary substitution that would be necessary for the subsequent 10 weeks of the study. Following the 2 week run-in phase, baseline (week 0) measures were obtained and subjects were randomly assigned to one of two groups which determined the order of the dietary crossover. The two dietary periods were “soy” (4 serves per day of soy milk and/or yoghurt) and “dairy” (4 serves per day of dairy low-fat milk and/or yoghurt). To aid in compliance, subjects were allowed the choice of incorporating either milk, yoghurt, or a combination of both in their diets. After 5 weeks on the first diet, the two groups crossed over to the alternate diet for the following 5 weeks.

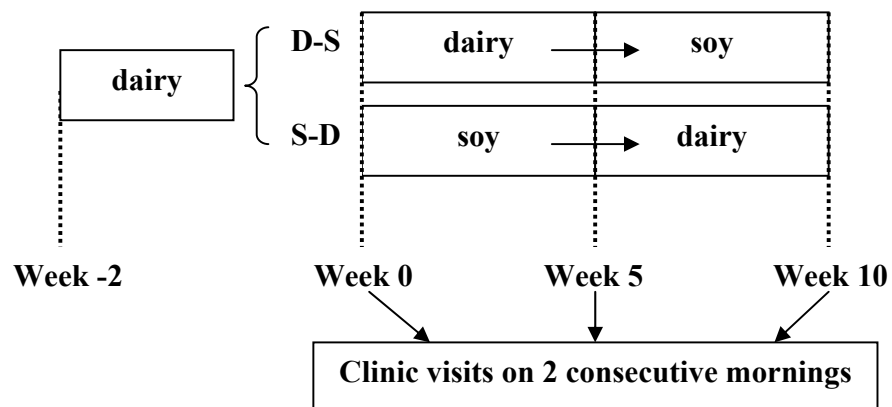


Figure 4.1 Study Design.

At the beginning of weeks 0, 5 and 10, subjects attended clinic visits on 2 consecutive mornings. On the first morning, height and weight were recorded, a dietary interview was conducted by a registered dietitian and clinic blood pressure and arterial compliance were measured. A fasted venous blood sample was taken by a qualified nurse for determination of plasma levels of isoflavones, lipids, fatty acids and antioxidant status. For the 24 h between the first and second visits, an ambulatory blood pressure monitor was worn (Model 90217, Spacelabs Medical Inc. Redmond WA, USA) and urine collected into 2.5 L vessels (each containing 1g ascorbic acid). On the second morning, a fasted blood sample was taken for repeat lipid assays, the completed 24 h urinary sample was handed in and blood pressure data were downloaded. Only results of plasma and urinary isoflavones and blood lipids are reported in this thesis.

4.2.3 Study foods

During the soy dietary period, subjects consumed soy milk (Ultra Heat Treated (UHT) Calciforte) and low fat (2%) soy yoghurt (both from So Natural Foods Australia Ltd, Taren Point, NSW, Australia). During the dairy dietary period, subjects consumed Lite-White reduced fat (1.4%) dairy milk (UHT, Dairy Farmers Ltd, Lidcombe, NSW, Australia) and low fat (1.5%) dairy yoghurt (LC1, Nestle Australia Ltd, Homebush,

NSW, Australia). All of the study foods, including those manufactured by other companies were provided by So Natural Foods. Subjects collected their study foods from the clinic in 2-3 week intervals and were instructed to keep them refrigerated. A 250 mL serve of soy milk provided 9.25 mg daidzein + 12.75 mg genistein and a 200 g serve of yoghurt provided 7.8 mg daidzein and 11.2 mg genistein (So Natural Foods). Subjects were instructed to consume 4 serves per day of either the soy or dairy foods provided, where 1 serve was equivalent to 250 mL milk or 200 g yoghurt.

4.2.4 Sample analysis

Immediately after each blood sample was taken it was placed on ice until the end of each morning of clinic visits, when all samples were centrifuged at 3000 rpm at 4°C (Hettich, Universal 16R) for 10 min. Plasma was removed, and aliquots stored at –80°C prior to analysis. After the volume of each 24 h urinary sample was measured and recorded, aliquots were also stored at –80°C until analysis. Cholesterol content was determined for total plasma, triglycerides and isolated HDL using the standard commercially available kits (Roche Diagnostic) and an autoanalyser (Cobas Mira Plus). Plasma LDL cholesterol was calculated using the Friedewald calculation (Friedewald *et al.*, 1972). Concentrations of daidzein, genistein, equol and ODMA in plasma and urine samples were extracted as described in 2.3.2 and quantified as described in 2.3.3.

4.2.5 Statistical analysis

All data were analysed for normal distribution and presence of outliers (SPSS version 11.5, Chicago, Illinois). To determine whether there was an order effect of the dietary periods on any measures, one-way ANOVAs with repeated measures (ANOVA/RM) and between groups analyses were performed. Combined groups were analysed via one-way ANOVA/RM and Bonferroni post-hoc analysis. Correlations are reported as Pearson's correlation coefficient (R). Means are reported as \pm standard error of the mean (SEM).

4.3 RESULTS

4.3.1 Subjects and compliance

Twenty-six subjects commenced the study, but three males withdrew during the first 5 weeks (one subject was uncomfortable with the 24 h ambulatory BP monitor, another could not incorporate the 4 serves of study foods into his diet and the third withdrew for personal reasons). Twenty three subjects (13 males and 10 females) completed the study, with a mean age at baseline of 54 ± 2 years. There were no significant changes in body weight or BMI during the study (Table 4.1).

Table 4.1 Anthropometric Data.

						ANOVA [†]			female vs. male [‡]
	subjects	n	baseline	dairy	soy	F	df	p	
mass (kg)	female	10	70.5 \pm 2.4	71.0 \pm 3.0	71.0 \pm 2.9	0.216	2,8	0.810	F = 6.516 p = 0.019
	male	13	80.1 \pm 2.3	80.2 \pm 2.5	80.7 \pm 2.6	2.605	2,11	0.119	
	all	23	75.9 \pm 1.9	76.2 \pm 2.1	76.5 \pm 2.2	1.936	2,21	0.169	
BMI (kg/m ²)	female	10	26.9 \pm 1.0	27.0 \pm 1.2	27.1 \pm 1.2	0.268	2,8	0.772	F = 0.946 p = 0.342
	male	13	25.6 \pm 0.7	25.7 \pm 0.8	25.8 \pm 0.8	2.483	2,11	0.129	
	all	23	26.2 \pm 0.6	26.3 \pm 0.7	26.4 \pm 0.7	1.864	2,21	0.180	

[†]One-way ANOVA with repeated measures. [‡]One-way ANOVA with repeated measures and between-groups analysis.

4.3.2 Treatment of data

There were no significant differences between the two groups in weight, BMI or plasma or urinary isoflavones, when baseline, dairy and soy were compared (Appendix 4.1). This indicated no effect of the order in which the two dietary phases were followed and thus, the same treatments of the two groups were combined for all isoflavone analyses.

At the end of the soy phase, isoflavone data was distributed normally and no values were identified as outliers.

4.3.3 Isoflavones at baseline and after dairy

The baseline plasma samples were mostly devoid of isoflavones with the exception of one subject, whose plasma contained 53.6 ng/mL genistein. Eleven subjects had small amounts of both daidzein and genistein in their baseline 24 h urine sample after the initial 2-week run-in phase, with a mean excretion for these subjects of 5.0 ± 0.6 mg (range 2.5 – 9.4 mg) daidzein and 2.2 ± 0.5 mg (range 0.9 – 7.1 mg) genistein. One of these subjects also excreted 2.8 mg ODMA. A different subject had very small amounts of both genistein and equol in their urine (<1 mg of each) but no daidzein.

After 5 weeks of dairy intake, isoflavones were evident in the plasma of four subjects and were excreted in the urine of ten subjects, with only one of these subjects having both plasma and urinary isoflavones at this time. Daidzein, genistein and equol were found in the plasma of one subject, daidzein only in two others, and equol only in one subject. The concentrations of these plasma isoflavones ranged from 15.6 – 27.2 ng/mL daidzein, and were 84.0 ng/mL genistein and 51.6 and 26.7 ng/mL equol. The urine of 7 subjects contained detectable levels of daidzein, while genistein was detected in the urine of 3 subjects, equol in the urine of 3 subjects and ODMA in the urine of 1 subject. Three subjects had more than one isoflavone in their overnight urinary excretion. The 24 h urinary isoflavone excretion after 5 weeks dairy intake ranged from 0.4 – 16.4 mg daidzein, 0.51 – 5.8 mg genistein and 0.6 – 4.2 mg equol.

4.3.4 Isoflavone levels after soy

Plasma daidzein and genistein were both significantly elevated after soy compared with both baseline and after dairy (Table 4.2). Four serves per day of the soy milk provided

37 mg daidzein and 51 mg genistein and of the soy yoghurt provided 31 mg daidzein and 45 mg genistein. There was one subject however, whose plasma was devoid of any isoflavones and two others whose plasma contained genistein only. The plasma level of genistein significantly exceeded that of daidzein for all subjects during the study ($F_{1,22} = 38.327$, $p < 0.0001$, two-way ANOVA/RM). The mean plasma daidzein: genistein (D/G) ratio was 0.34 ± 0.05 and plasma daidzein and genistein concentrations after soy intake were correlated ($R = 0.456$, $p = 0.05$, $n = 23$). The only subject to have measurable plasma equol after soy (32 ng/mL) also had the highest plasma concentration of daidzein. ODMA was present in 2 subjects (31 ng/mL and 104 ng/mL) who both also had plasma daidzein levels greater than the mean. The limited occurrence of equol and ODMA in plasma did not allow for statistical comparison between the diets.

Table 4.2 Mean plasma and urinary isoflavones at baseline and after dairy and soy. Mean \pm SEM, $n = 23$.

	treatment [†]			ANOVA [‡]	
	baseline	dairy	soy	F _{2,21}	p
Plasma (ng/mL)					
daidzein	0 ^a	2.6 ± 1.5 (0 - 27.2) ^a	62.1 ± 8.4 (0 - 164) ^{b*}	25.984	<0.0001
genistein	2.3 ± 2.3 (0 - 53.4) ^a	3.7 ± 3.7 (0 - 84.0) ^a	257 ± 33.8 (0 - 533) ^{b*}	27.664	<0.0001
equol	0.0 ± 0.0 (0 - 0.3)	3.4 ± 2.5 (0 - 51.6)	1.4 ± 1.4 (0 - 32.1)	-	-
O-DMA	0	0	8.1 ± 5.7 (0 - 104)	-	-
Urine (mg)					
daidzein	2.4 ± 0.6 (0 - 9.4) ^a	1.3 ± 0.7 (0 - 16.4) ^a	12.4 ± 1.5 (0 - 25.9) ^{b*}	21.855	<0.0001
genistein	1.1 ± 0.3 (0 - 7.1) ^a	0.3 ± 0.3 (0 - 5.8) ^a	9.5 ± 1.2 (0 - 21.3) ^{b*}	26.797	<0.0001
equol	0.0 ± 0.0 (0 - 0.4)	0.2 ± 0.2 (0 - 4.2)	0.2 ± 0.2 (0 - 5.3)	-	-
O-DMA	0.1 ± 0.1 (0 - 2.8) ^a	0.1 ± 0.1 (0 - 1.3) ^a	3.1 ± 0.6 (0 - 9.9) ^b	13.854	<0.0001

[†]Values with different superscripts are significantly different ($p < 0.05$, $*p < 0.01$).

[‡]One-way ANOVA with repeated measures.

After soy, both daidzein and genistein were present in the urine of 22 of the 23 subjects. There was a significant effect of diet on the urinary excretion of both isoflavones, due to significantly elevated levels after soy compared with both baseline and dairy (Table 4.2). The subject whose plasma was devoid of isoflavones after soy also did not excrete any

isoflavones in their urine. Urinary daidzein was significantly greater than urinary genistein during the study ($F_{1,22} = 17.574$, $p < 0.0001$, two-way ANOVA/RM), with a mean urinary D/G ratio of 1.43 ± 0.12 and significant correlation between daidzein and genistein ($R = 0.875$, $p = 0.01$, $n = 23$).

Sixteen subjects (70%) excreted ODMA after 5 weeks of soy intake, with a mean 24 h excretion of 4.5 ± 0.5 mg. There was a significant effect of diet as only one of these subjects excreted this daidzein metabolite at baseline and after dairy (Table 4.3). Urinary excretion of ODMA after soy was correlated with that of daidzein ($R = 0.633$, $p = 0.01$, $n = 23$, Figure 4.2). The only 2 subjects with ODMA in their plasma after soy (83.13 ng/mL and 104.29 ng/mL, respectively) also excreted the largest amounts of 24 h urinary ODMA (6.9 mg and 9.9 mg, respectively). Equol was excreted by one subject (5.3 mg/24 h) who excreted one of the lower amounts of daidzein.

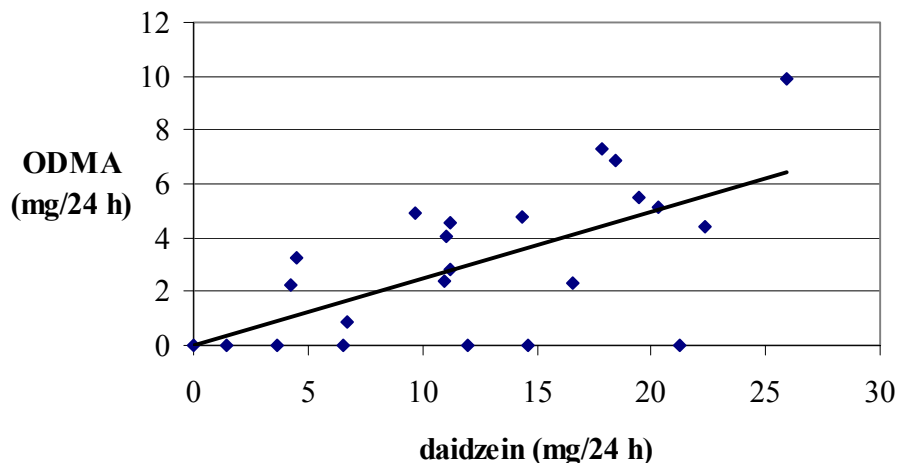


Figure 4.2 Correlation between urinary excretion of daidzein and ODMA after 5-week soy dietary period. $R = 0.633$, $p = 0.01$, $n = 23$.

As equol appeared sporadically in the plasma of 3 subjects and the urine of 5 other subjects, correlations between this metabolite and other isoflavones were not possible. Consequently, subjects were divided into “equol-positive” or “equol-negative” on the basis of equol being present in either their plasma or urine in *any sample* during the study. There were no significant differences between equol-positive and equol-negative subjects for any other plasma or urinary isoflavones during the study (Table 4.3). Four of the equol-positive subjects (2 female and 2 male) were among the seven subjects who did not excrete ODMA after soy and in addition, the 2 female subjects had no or very little plasma or urinary daidzein. In contrast, the other four equol-positive subjects excreted greater than the average amount of ODMA after soy.

Table 4.3 Mean plasma and urinary isoflavones for equol-positive and equol-negative subjects. n = 8 and n = 15, respectively.

			equol	baseline	dairy	soy	F	p [†]
plasma (ng/mL)	daidzein	+ ve		0	2.0 ± 2.0	57.8 ± 19.1	0.152	0.700
		- ve		0	2.9 ± 2.0	64.3 ± 8.5		
	genistein	+ ve		0	10.5 ± 10.5	262 ± 72.9	0.038	0.847
		- ve		3.6 ± 3.6	0	254 ± 36.6		
	ODMA	+ ve		0	0	0	1.107	0.305
		- ve		0	0	12.5 ± 8.6		
urine (mg/24 h)	daidzein	+ ve		1.7 ± 0.90	0.71 ± 0.65	11.4 ± 2.8	0.785	0.386
		- ve		2.7 ± 0.81	1.6 ± 1.1	12.9 ± 1.8		
	genistein	+ ve		0.84 ± 0.35	0.14 ± 0.09	8.4 ± 2.1	0.728	0.403
		- ve		1.2 ± 0.48	0.38 ± 0.38	10.1 ± 1.5		
	ODMA	+ ve		0	0	2.8 ± 1.1	0.368	0.551
		- ve		0.19 ± 0.19	0.09 ± 0.09	3.3 ± 0.71		

†One way ANOVA with repeated measures and between groups analysis.

4.3.5 Gender differences in isoflavone bioavailability

Plasma genistein was significantly higher in males than females during the study (Table 4.4) and there was an interaction between gender and diet for plasma daidzein ($F_{2,20} = 3.881$, $p = 0.038$, one-way ANOVA/RM), due to males having higher plasma daidzein than females after soy, which approached significance ($p = 0.052$, Student's paired t-tests with a Bonferroni-adjusted level of significance of $p = 0.017$). The 24 h urinary excretion of daidzein and genistein was consistently higher in males; this was significant for genistein and approached significance for daidzein (Table 4.5). There was no gender difference for plasma or urinary ODMA (Table 4.5).

Table 4.4 Mean plasma and urinary daidzein and genistein for females and males. f = female, n = 10; m = male, n = 13.

gender			baseline	dairy	soy	F	p [†]
plasma (ng/mL)	daidzein	f	0	4.3 ± 3.0	43.6 ± 10.1	2.955	0.100
		m	0	1.2 ± 1.2	76.3 ± 11.5		
	genistein	f	0	0	165 ± 32.3	8.134	0.010
		m	4.1 ± 4.1	6.5 ± 6.5	328 ± 46.3		
	ODMA	f	0	0	8.3 ± 8.3	0.001	0.980
		m	0	0	8.0 ± 8.0		
urine (mg/24 h)	daidzein	f	2.3 ± 0.96	0.79 ± 0.92	9.1 ± 2.2	4.060	0.057
		m	2.5 ± 0.82	1.7 ± 1.3	14.9 ± 1.8		
	genistein	f	0.58 ± 0.26	0	7.0 ± 1.8	6.529	0.018
		m	1.5 ± 0.54	0.53 ± 0.44	11.4 ± 1.5		
	ODMA	f	0.28 ± 0.28	0.13 ± 0.13	2.9 ± 0.76	0.014	0.908
		m	0	0	3.2 ± 0.88		

†One way ANOVA with repeated measures and between groups analysis.

4.3.6 Dietary effects on plasma lipids

There were no significant differences between the two study groups for total, HDL or LDL cholesterol ($F = 1.190$, $p = 0.288$; $F = 0.885$, $p = 0.358$ and $F = 1.809$, $p = 0.193$ respectively, one-way ANOVA/RM and between groups analysis, Appendix 4.2), however, there was an interaction between group and triglycerides ($F_{2,20} = 6.293$, $p = 0.008$, Appendix 4.2). Subsequently, the two groups were combined for the analysis of total cholesterol (TCh), HDL and LDL, but analysed separately for triglycerides (TG).

When the two groups were combined, there were no significant effects of diet on total, HDL or LDL cholesterol (Table 4.5). There was an overall effect of diet on TG that approached significance for both groups, but this appeared to be due to a temporal rather than a diet effect. For both groups, the largest mean change in TG was between weeks 5 and 10 and this approached significance (Group 1: $p = 0.082$, Group 2: $p = 0.075$, Bonferroni post-hoc tests). However, this was during the soy dietary period for Group 1 and the dairy dietary period for Group 2.

Table 4.5 Mean lipid levels at baseline and after dairy and soy for combined groups. $n = 23$. One-way ANOVA with repeated measures. TCh = total cholesterol, HDL = HDL-cholesterol, LDL = LDL-cholesterol, TG = triglycerides.

	Lipids (mmol/L)			ANOVA		
	baseline	dairy	soy	F	df	p
TCh	5.70 ± 0.23	5.59 ± 0.22	5.54 ± 0.20	0.543	2,21	0.589
HDL	1.14 ± 0.07	1.09 ± 0.05	1.14 ± 0.07	1.181	2,21	0.326
LDL	3.99 ± 0.24	3.93 ± 0.20	3.85 ± 0.20	0.628	2,21	0.543
	Week 0	Week 5	Week 10			
TG (Group D-S)	1.23 ± 0.14	(dairy) 1.39 ± 0.20	(soy) 1.09 ± 0.12	3.349	2,10	0.077
TG (Group S-D)	1.38 ± 0.26	(soy) 1.37 ± 0.24	(dairy) 1.09 ± 0.17	3.222	2,9	0.088

4.3.7 Correlations between baseline lipids and subsequent changes in lipids

To determine whether baseline lipid measures were a determinant of any lipid changes due to diet, correlations were performed between baseline lipids and the effects of dairy and soy (the differences between baseline and the end of the dairy or soy dietary periods; Table 4.6). Baseline TCh was inversely correlated with the change in both TCh and LDL during the soy dietary period, whilst baseline LDL was inversely correlated with the changes in TCh and LDL during both the dairy and the soy dietary periods (Figure 4.3). Baseline HDL and TG were inversely correlated with the changes in HDL and TG, respectively, with the dairy dietary period.

Table 4.6 Correlations between baseline lipids (mmol/L) and lipid changes (mmol/L) with dairy or soy. TCh = total cholesterol, HDL = high density lipoprotein, LDL = low-density lipoprotein, TG = triglycerides. R = Pearson's correlation coefficient.

		TCh		HDL		LDL		TG	
		R	p	R	p	R	p	R	p
Δ TCh	dairy	-0.340	0.112	0.244	0.262	-0.464	0.026	0.267	0.218
	soy	-0.482	0.020	0.105	0.633	-0.560	0.005	0.274	0.207
Δ HDL	dairy	0.240	0.270	-0.632	0.001	0.321	0.135	0.321	0.136
	soy	-0.158	0.471	-0.339	0.113	-0.080	0.718	0.099	0.654
Δ LDL	dairy	-0.375	0.078	0.373	0.080	-0.537	0.008	0.281	0.194
	soy	-0.430	0.041	0.240	0.271	-0.564	0.005	0.331	0.123
Δ TG	dairy	0.064	0.772	-0.023	0.917	0.182	0.405	-0.428	0.042
	soy	-0.177	0.419	-0.149	0.497	-0.027	0.903	-0.365	0.087

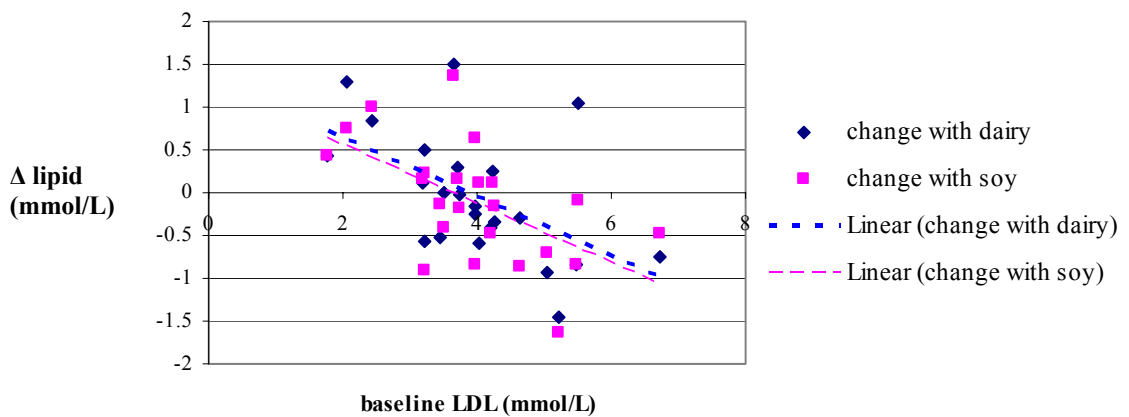
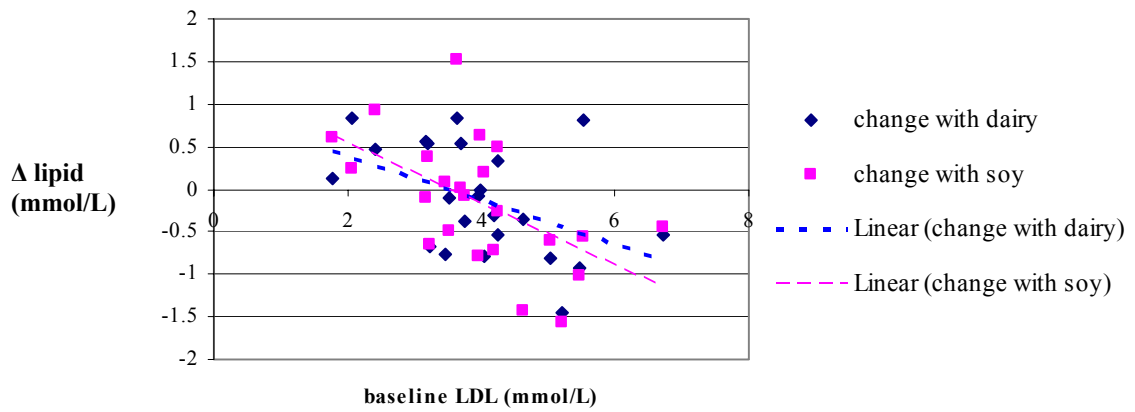
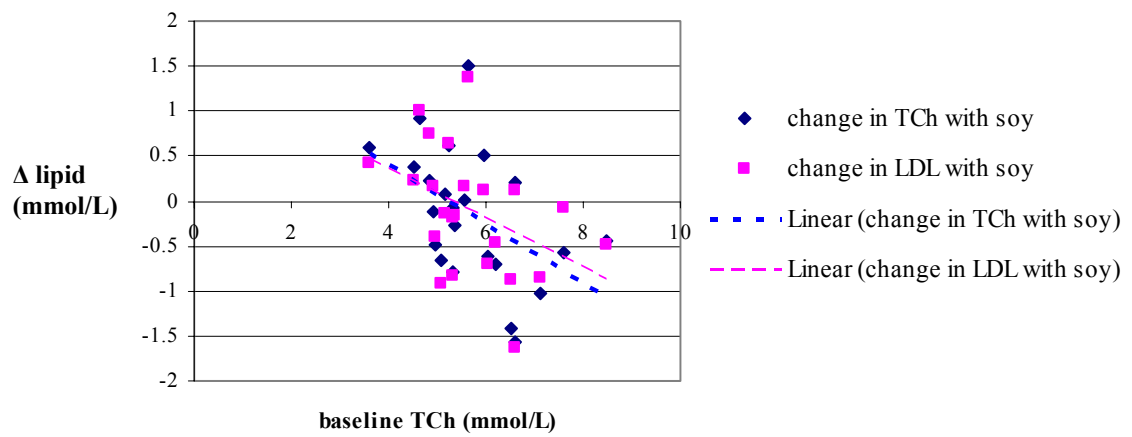


Figure 4.3 Correlations between baseline lipids and the subsequent change in lipid levels with soy or dairy dietary periods.

4.3.8 Isoflavones and lipid levels

At the end of the soy dietary period, there were no significant correlations between any plasma or urinary isoflavones and any lipid changes, although a positive correlation between plasma daidzein and an increase in HDL approached significance (Table 4.7).

Table 4.7 Correlations between plasma and urinary isoflavones and changes in lipids with soy dietary period.

		TCh		HDL		LDL		TG	
		R	p	R	p	R	p	R	p
plasma	daidzein	0.334	0.119	0.405	0.056	0.286	0.186	-0.266	0.221
	genistein	0.083	0.708	0.245	0.260	0.078	0.722	-0.330	0.124
	ODMA	0.199	0.364	0.291	0.177	0.103	0.641	0.114	0.606
urine	daidzein	-0.029	0.897	0.116	0.598	-0.070	0.750	0.029	0.894
	genistein	-0.006	0.978	-0.101	0.646	0.023	0.917	0.001	0.997
	ODMA	-0.049	0.823	0.267	0.218	-0.122	0.578	-0.046	0.837

However, when subjects were classified according to their “equol-status” (4.2.6), there were significant effects of diet on TCh and LDL for equol-positive subjects, but no effects for equol-negative subjects (Table 4.8). For equol-positive subjects, TCh was significantly reduced after soy compared with both baseline and dairy and there was a reduction in LDL after soy compared with dairy that approached significance ($p = 0.076$, Bonferroni post-hoc analysis). In addition, equol-positive subjects had a nearly significant reduction in mean TG after soy compared with dairy ($p = 0.067$, Bonferroni post-hoc analysis; Table 4.8). In contrast, the only effect of diet on lipids that approached significance in equol-negative subjects was a trend for increased TCh levels after soy compared with dairy ($p = 0.056$, Bonferroni post-hoc analysis).

Table 4.8 Mean lipid levels of equol-positive and equol-negative subjects at baseline and after dairy and soy.

	equol	Lipid Levels (mmol/L)*			ANOVA [†]			Between groups [‡]	
		baseline	dairy	soy	F	df	p	F	p
TCh	+ve	5.96 ± 0.33 ^a	5.91 ± 0.39 ^a	5.26 ± 0.29 ^b	15.168	2,6	0.005	0.110	0.743
	-ve	5.57 ± 0.30	5.42 ± 0.27	5.70 ± 0.27	3.337	2,13	0.068		
HDL	+ve	1.14 ± 0.09	1.11 ± 0.08	1.00 ± 0.08	1.554	2,6	0.286	0.269	0.609
	-ve	1.15 ± 0.09	1.08 ± 0.07	1.22 ± 0.09	2.805	2,13	0.097		
LDL	+ve	4.24 ± 0.29	4.12 ± 0.36	3.74 ± 0.26	5.57	2,6	0.043	0.183	0.673
	-ve	3.82 ± 0.33	3.83 ± 0.25	3.90 ± 0.27	0.364	2,13	0.702		
TG	+ve	1.27 ± 0.11	1.49 ± 0.16	1.12 ± 0.09	4.046	2,6	0.077	0.041	0.842
	-ve	1.32 ± 0.21	1.12 ± 0.18	1.28 ± 0.20	1.501	2,13	0.259		

*Superscripts with different letters are significantly different (p<0.05, Bonferroni post-hoc analysis).

[†]One-way ANOVA with repeated measures.

[‡]One way ANOVA with repeated measures and between groups analysis.

4.4 DISCUSSION

4.4.1 Plasma and urinary isoflavone levels after 5 weeks soy intake

In the current study, four serves per day of the whole-bean soy milk and/or yoghurt provided subjects with a daily intake of between 76 and 88 mg total isoflavones (daidzein + genistein); this equated to a mean intake of 0.45 ± 0.01 mg daidzein and 0.63 ± 0.02 mg genistein per kg body weight. After 5 weeks of this soy-enhanced diet, mean plasma levels of daidzein and genistein were 62.1 ± 8.4 ng/mL and 257 ± 33.8 ng/mL respectively, and mean 24 h urinary excretion was 12.4 ± 1.5 mg daidzein and 9.5 ± 1.2 mg genistein.

During the study, subjects not restricted as to when they consumed the 4 serves of soy each day and the plasma samples were not timed in relation to the last soy intake. However, as plasma samples were taken in the morning after an overnight fast, they would have occurred between 12 and 24 hours after the last dose. The mean ratio of daidzein: genistein in plasma was 0.34 ± 0.05 , indicating that plasma levels of genistein were approximately three times higher than those of daidzein, however it is common for genistein to be at higher levels in the plasma than daidzein (King and Bursill, 1998; Setchell *et al.*, 2003b). The faster plasma clearance rate of daidzein compared with genistein (Setchell *et al.*, 2003a; Shelnutt *et al.*, 2002) may have contributed to the relatively lower plasma levels of daidzein in this study, particularly in relation to the time elapsed (at least 12 hours) since the last soy intake. Conversely, this may suggest increased genistein bioavailability from whole soy bean foods or soy milk. When 4 healthy adult men were also fed 1 L soy milk per day for 4 weeks, the mean plasma levels of daidzein and genistein were 100 ng/mL and 350 ng/mL respectively (Mitchell and Collins, 1999) and although the isoflavone content of the soy milk was not reported in this study, the resulting plasma D/G ratio (0.29) is similar to that of the current study. The conversion of daidzein to ODMA, although the latter was not prevalent in plasma, may also have contributed to a decrease in the apparent plasma bioavailability of daidzein compared with genistein in the present study.

The levels of plasma daidzein and genistein in the current study are similar to those from other studies. Using similar isoflavone doses to the current study (0.47 mg daidzein and 0.59 mg genistein/kg body weight from soy milk powder), plasma levels 24 h after a single dose were 51 ng/mL daidzein and approximately 120 ng/mL genistein (Zhang *et al.*, 1999), a similar level of daidzein to the current study, though lower genistein. After 28 days of soy protein drink powder that provided a similar level of genistein (80.3 mg) to the current study but less daidzein (35.6 mg), plasma levels were higher in daidzein (127 ng/mL) but of a comparable genistein content (245 ng/mL) (Gooderham *et al.*, 1996). It is likely that the plasma isoflavone levels of subjects in the current study were higher during the day than after an overnight fast. Six hours after soy milk powder intake as above, Zhang and co-authors (1999) reported mean plasma levels of approximately 290 ng/mL daidzein and 475 ng/mL genistein, greater than the corresponding isoflavone levels 24 h after intake. Similarly, increased isoflavone bioavailability was expected during the day in the study reported here.

The mean 24 h urinary excretions of 12.4 ± 1.5 mg daidzein and 9.5 ± 1.2 mg genistein represent recoveries of approximately 36% daidzein and 20% genistein, although the exact amount would depend on the precise proportions in which subjects consumed the yoghurt or the milk. These values are in the range of recoveries reported in other studies following single intakes of various soy foods (TVP, tempeh, cooked soybeans, tofu and soymilk powder), of 20 - 60% for daidzein and 10 - 25% for genistein, (Xu *et al.*, 1994; Xu *et al.*, 2000; Zhang *et al.*, 1999; Zhang *et al.*, 2003). However, recoveries after a single meal may be greater than after chronic soy intake; Lu and co-authors (1996) reported a decrease in the percent isoflavone recovery after subjects were fed 1 L soy milk for 4 weeks from 66% to 45% for daidzein and 24% to 14% for genistein. This suggests that chronic intake may increase the metabolism of daidzein and genistein to other compounds or increase their tissue distribution. In the current study, neither daidzein nor genistein were correlated between plasma and urine, which may suggest that there are varying pathways of metabolism, possibly to end compounds that were not measured. In addition, tissue retention of isoflavones would affect their bioavailability. Furthermore, although the plasma level of genistein was greater than that of daidzein,

there was a higher recovery of daidzein in urine, suggesting greater biotransformation of genistein.

4.4.2 Plasma and urinary isoflavones at baseline and after 5 weeks dairy intake

At baseline and after dairy, the average background levels of isoflavones were approximately 3 ng/mL each of daidzein, genistein and equol in plasma, and approximately 2 mg daidzein and less than 1 mg genistein, equol and ODMA in urine. These plasma levels in the current study are similar to those reported for healthy men and women in the U.K. (mean 4.6 ng/mL daidzein and 6.5 ng/mL genistein; Wiseman *et al.*, 2004) and omnivorous Finnish women (mean 2.3 ng/mL daidzein and 1.6 ng/mL genistein; Adlercreutz *et al.*, 1993) but are less than those reported for postmenopausal women in Italy (mean plasma levels of 7.4 ng/mL daidzein and 17.5 ng/mL genistein; Albertazzi *et al.*, 1999) and in the U.S. (mean 16.5 ng/mL daidzein and 22 ng/mL genistein; Nettleton *et al.*, 2004). The background plasma level of equol was in the range of reported values of mean levels less than 1 ng/mL (Adlercreutz *et al.*, 1993; Nettleton *et al.*, 2004; Wiseman *et al.*, 2004), but with some individuals having levels up to 51 ng/mL (Albertazzi *et al.*, 1999). In a variety of other studies of subjects consuming their habitual diet in Australia (Dalais *et al.*, 1998) or other diets in America (fruit and vegetable, legume, carotenoid or cruciferous, subjects have excreted less than 1 mg daidzein or genistein or equol per day (Hutchins *et al.*, 1995a; Hutchins *et al.*, 1995b; Karr *et al.*, 1997; Kirkman *et al.*, 1995; Wiseman *et al.*, 2004). In the current study, levels at baseline and after dairy treatment represent those of a diet high in dairy products, which may contribute an alternative source of isoflavone precursors to the diet. However, King and co-authors (1998) reported that the concentration of isoflavones in Australian milk samples are extremely low.

4.4.3 Gender differences in isoflavone bioavailability

In the present study, there were some gender differences in isoflavone bioavailability. After the 5-week soy dietary period, males had significantly higher plasma and urinary genistein and a trend towards greater plasma and urinary daidzein compared with females. During the soy intervention, isoflavones were not administered as specific doses according to body weight, and thus differences in body mass would have contributed to the disparity between male and female isoflavone levels. However, the mean isoflavone dose per kg was lower in males than females (0.43 ± 0.01 mg daidzein and 0.61 ± 0.02 mg genistein per kg body mass compared with 0.49 ± 0.02 mg daidzein and 0.69 ± 0.03 mg genistein per kg body mass, respectively), the opposite of the observed higher plasma and urinary isoflavone levels in males. Females ingested a proportionally higher amount relative to their body mass than men, but had lower levels in plasma and urine, suggesting that a greater proportion of the isoflavones may be distributed to tissue, perhaps as a result of higher tissue density of estrogen receptors. Maubach and co-authors (2003) suggested that isoflavones could occupy estrogen receptor sites or accumulate in lipophilic tissues such as breast tissue, in which the authors quantified isoflavone levels.

After 5 weeks of daily soy intake in the current study, the mean plasma isoflavone levels in females and males were 43.6 ± 10.1 ng/mL and 76.3 ± 11.5 ng/mL daidzein respectively and 165 ± 32.3 ng/mL and 328 ± 46.3 ng/mL genistein, respectively. After a single dose of soy milk powder in 7 females and 7 males providing 0.47 mg daidzein and 0.59 mg genistein per kg body weight, Zhang and colleagues (1999) also reported higher mean plasma genistein 24 h post-intake in males (135 ng/mL) compared with females (108 ng/mL) but no difference in plasma daidzein. At 6 h post-intake, these authors reported greater disparity between genders for daidzein (330 ng/mL in males compared with 254 ng/mL in females) than genistein (486 ng/mL in males compared with 459 ng/mL in females). These gender-related effects may be due to different pharmacokinetics of absorption and excretion rather than differences in absolute

absorption. In addition, since isoflavones undergo enterohepatic circulation similar to endogenous steroids (Adlercreutz *et al.*, 1987), endogenous hormone levels may affect the enterohepatic circulation of isoflavones, and thus their bioavailability. Although the ranges of urinary excretion of daidzein and genistein did not differ between males and females after daily intake of 1 L soy milk for 4 weeks, (Lu *et al.*, 1995; Lu *et al.*, 1996), the elimination half lives of both daidzein and genistein were greater in women and the absorption half life of genistein was also slightly longer in women than men (Lu and Anderson, 1998). However, longer pharmacokinetic isoflavone profiles in females would be expected to result in greater 24 h isoflavone levels; the opposite to this effect was observed in the current study.

In contrast to the gender differences in plasma and urinary daidzein and genistein in the current study, the plasma and urinary levels of ODMA were very similar between genders. If females originally absorbed less daidzein, this may imply that they have a more efficient conversion to ODMA. Conversely the gender difference may not be related to the initial absorption of the isoflavones, but rather to tissue distribution and/or sequestration. In the latter case, females may have greater tissue sequestration of isoflavones which could possibly be explained by a greater density of estrogen receptors (Maubach *et al.*, 2003). Further elucidation of this effect and determination of whether this gender difference is also evident in premenopausal women may help to ascertain whether isoflavone bioavailability is determined by endogenous hormone status and whether tissue distribution of isoflavones is related to estrogen receptor distribution.

4.4.4 Effects of soy diet on lipids

In the current study, 5 weeks of daily consumption of soy milk and/or yoghurt did not result in any significant lipid effects in 23 mildly hyperlipidemic subjects when compared with either baseline or the dairy control (Table 4.5). The mean changes during the 5-week soy dietary period were -0.14 ± 0.15 mmol/L TCh (1.6%), -0.11 ± 0.13 mmol/L LDL (-1.4%), $+0.04 \pm 0.05$ mmol/L HDL (4.7%) and -0.16 ± 0.08 mmol/L TG

(4.1%). These lipid changes are of a similar magnitude to those reported in two recent meta-analyses of studies that used similar intakes of soy protein and isoflavones in subjects with baseline total cholesterol between 4.57 and 6.60 mmol/L (Harland and Carr, 2004; Weggemans and Trautwein, 2003). However, similar studies providing soy protein and isoflavone levels comparable to those in the current study (25 - 43.5 g and 56 - 132 mg, respectively) in subjects with baseline TCh between 3.9 and 7.4 mmol/L have reported a range of results in terms of lipid effects. No lipid effects (Gooderham *et al.*, 1996; Kreijkamp-Kaspers *et al.*, 2004; Mitchell and Collins, 1999; Steinberg *et al.*, 2003), non-significant mean decreases in TCh and LDL (Baum *et al.*, 1998; Gardner *et al.*, 2001; Tonstad *et al.*, 2002) and significant decreases in TCh and LDL and increases in HDL (Bricarello *et al.*, 2004; Crouse *et al.*, 1999; Cuevas *et al.*, 2003; Potter *et al.*, 1998) have been reported. Thus, the effects of soy on lipids remain controversial and appear to be influenced by other factors.

In the current study, baseline TCh was significantly inversely correlated with the change in TCh and LDL during the soy diet period (Figure 4.3) and the regression line crossed the x-axis at a baseline TCh level close to 6.0 mmol/L. This is comparable with a trend observed in the studies mentioned above, of which only those subjects with an initial mean TCh >6.0 mmol/L demonstrated mean lipid lowering effects. The original meta-analysis of soy protein and lipids (Anderson *et al.*, 1995) also demonstrated greater lipid-lowering effects of soy in subjects with more elevated TCh. Baseline LDL in the present study was also correlated with the changes in LDL during both soy and dairy dietary periods and there was a distinction between positive and negative effects at a baseline LDL level of 4.0 mmol/L (Figure 4.3), similar to that reported previously (Crouse *et al.*, 1999). Thus overall, the findings of the current study, and others, indicate a strong influence of initial TCh and LDL levels on the lipid-lowering effects of soy intake; this finding will be further discussed in Chapter 7.

With 5 weeks of the soy diet there was a mean increase in HDL of 0.04 ± 0.05 mmol/L (4.7%) while this decreased by 0.07 ± 0.04 mmol/L (4.3%) with dairy. There was a trend towards a beneficial increase in HDL in this study regardless of initial lipid levels,

though this was not statistically significant. Other researchers have reported increases in HDL with soy of between 0.065 and 0.10 mmol/L (Baum *et al.*, 1998; Bricarello *et al.*, 2004; Gardner *et al.*, 2001; Potter *et al.*, 1998; Tonstad *et al.*, 2002) while others have reported no changes (Crouse *et al.*, 1999; Gooderham *et al.*, 1996; Kreijkamp-Kaspers *et al.*, 2004) or a decrease of 0.06 mmol/L (Steinberg *et al.*, 2003).

There was also a trend for TG to decrease more with the soy diet than with dairy; -0.16 ± 0.08 mmol/L (4.1%) compared with -0.05 ± 0.07 mmol/L (1.6%). While TG is mostly unchanged by many soy treatments (Bricarello *et al.*, 2004; Crouse *et al.*, 1999; Gardner *et al.*, 2001; Kreijkamp-Kaspers *et al.*, 2004; Mitchell and Collins, 1999; Steinberg *et al.*, 2003), some have reported small non-significant decreases of 0.16 mmol/L (Baum *et al.*, 1998; Tonstad *et al.*, 2002). In the study with the highest baseline triglyceride levels (2.15 mmol/L), a significant decrease of 0.62 mmol/L was reported (Cuevas *et al.*, 2003). However, in the current study there seemed to be a greater influence of overall study duration than dietary treatment (soy or dairy) on triglyceride levels. Both groups of the crossover showed an effect over time rather than of treatment with a mean decrease of 0.20 ± 0.14 mmol/L from weeks 5 – 10. The effects of soy on TG levels may take longer than 5 weeks as Tonstad and co-authors (2002) reported that with soy protein intake, TG levels continued to decrease for 8 weeks of supplementation and Baum and colleagues (1998) reported that TG levels were at their lowest after 12 or 24 weeks of soy protein and isoflavone supplementation. In the current study, there was a similar effect of time in both groups regardless of intervention order, suggesting that dairy yoghurt and/or milk may have an influence on TG levels. Conversely this effect may be related to lifestyle changes from participation in a dietary study. While no effects on TG levels were reported with the intake of a fermented milk product (Agerbaek *et al.*, 1995) or with dairy milk (Mitchell and Collins, 1999), the control treatments in soy interventions (non-fat dairy milk and milk and casein proteins) have produced decreases of up to 0.17 mmol/L TG after 4 – 8 weeks (Baum *et al.*, 1998; Gardner *et al.*, 2001; Tonstad *et al.*, 2002). In addition, because there are large day to day variations in TG levels (Taylor and Williams, 1998), it may be difficult to detect a significant change.

4.4.5 Metabolism to ODMA and equol

Of the two daidzein metabolites measured (equol and ODMA), ODMA was more prevalent, present in the urine of 16 subjects (70%) after the soy dietary period. Equol was only present in the plasma or urine of 8 subjects (35%), suggesting that the conditions of this study were not conducive to metabolism of daidzein to equol, although this occurrence of equol is similar to other studies (Lampe *et al.*, 1998; Lampe *et al.*, 2001; Slavin *et al.*, 1998). Considering that the plasma samples were taken at least 12 h after the last intake of soy and that the 24 h urine samples were collected at the end of 5 weeks of daily soy intake, there was ample opportunity for the conversion of daidzein to equol prior to sampling. The sporadic occurrence of equol and the lack of its presence in both plasma and urine of the same subject did not allow for comprehensive further analysis. Although Wiseman and co-authors (2004) noted reduced plasma genistein levels in equol-positive subjects compared with equol negative subjects with chronic soy consumption, there was no difference between equol-positive and equol-negative subjects in the current study for plasma or urinary levels of daidzein or genistein. The finding here is in agreement with that of Lampe and co-authors (2001), who reported similar urinary daidzein and genistein excretion independent of equol-producing status.

4.4.6 Influence of equol on lipid effects

When subjects were classified according to equol status, the equol-positive subgroup had a higher mean baseline cholesterol level and this may have contributed to the subsequent significant decreases in TCh and LDL with soy intake. However, half of these subjects had a baseline TCh <6 mmol/L, thus this sub-groups was representative of the entire group in terms of initial TCh and its potential influence on subsequent lipid effects of soy intake. Within the “equol-positive” subgroup, there was an additional influence of baseline TCh. Although the soy dietary period resulted in a mean reduction in TCh of 7% for all equol-positive subjects, there was a mean reduction of 1.07 mmol/L (16%) in equol-positive subjects with baseline TCh >6mmol/L and of 0.34 mmol/L (6.5%) in

equol-positive subjects with baseline TCh <6mmol/L. However, as there was no correlation between lipid changes and plasma or urinary equol levels, it appears that equol itself is not responsible for the lipid lowering effects. Rather, equol production may be a marker of a particular phenotype for responsiveness to soy supplementation or of a specific gut microfloral profile (Lampe *et al.*, 1998) that may facilitate the lipid lowering effects of the isoflavones and/or soy protein components of soy foods. Indeed, gut microfloral activity and in particular, the short chain fatty acids produced by bacterial fermentation, can affect serum lipids. Jenkins and co-authors (1999) reported that differences between individuals in gut microfloral activity can account for up to 30% of variation in lipid levels and that the ratio of particular bacteria species is related to serum levels of LDL cholesterol. Furthermore, acetate produced during carbohydrate fermentation can increase, while propionate can decrease, serum cholesterol (Jenkins *et al.*, 1999).

4.4.7 Inter-individual variation and study design

Because the design of this study did not stipulate that subjects record the time of consumption of soy each day, plasma isoflavone levels could not be related to the time since the last intake of soy food. Nonetheless, they still represent habitual consumption from the previous day. There was large inter-individual variation in the plasma and urinary levels of isoflavones, a common feature of human isoflavone bioavailability studies. The incorporation of 4 serves per day of soy or dairy milk and/or yoghurt is higher than the usual intakes of these foods in consumers of a Western diet and may not represent a normal level of inclusion of soy foods in the diet. Although there were no significant differences between the two groups in their plasma or urinary isoflavones after the 5 weeks of daily soy supplementation, there was a tendency for greater urinary excretion of both daidzein and genistein after the soy phase in the group who followed this dietary period first (daidzein 14.0 ± 8.2 mg/24 h compared with 10.8 ± 5.9 mg/24 h and genistein: 11.6 ± 6.9 mg/24 h compared with 7.6 ± 3.9 mg/24 h) which may indicate decreased compliance when the soy foods were consumed during the second 5 weeks.

Further, the order of treatment may have especially been important in relation to lipid effects as there was no washout between the two dietary periods. Thus, study design could be improved with the inclusion of a washout period between the two crossover diets.

4.4.8 Conclusions and future directions

The daily consumption of whole soy bean milk significantly increased isoflavone bioavailability in plasma and urine, even up to 24 h after the last soy milk intake. There were some gender differences that suggest that endogenous hormones may affect the pharmacokinetics of isoflavones, including the potential for increased production of ODMA relative to daidzein in females. Despite the overall lack of significant effect of 5 weeks whole soy bean milk supplementation on lipid levels in this study population, there was a significant influence of baseline TCh and LDL levels and of equol production on the subsequent lipid changes with soy intake. Although it does not appear that the conversion of daidzein to equol was the mechanism by which soy produced significant lipid lowering effects in these subjects, further work to examine this post-hoc result would be helpful in determining the potential actions of soy and its isoflavones in relation to lipid effects. Thus, a dietary intervention in hyperlipidemic subjects using a combination of soy and a prebiotic (shown to increase equol production in Chapter 3) is proposed to increase equol production and possibly enhance lipid lowering benefits (Chapter 5). Alternatively, equol and daidzein could be provided independently, with and without soy protein isolate to determine their individual roles in the purported hypocholesterolemic effects of soy.

4.5 APPENDIX

Appendix 4.1 Comparison between the two study groups for plasma and urinary isoflavones, weight and BMI.

		Group	baseline	dairy	soy	F	p [†]
Plasma (ng/mL)	daidzein	D-S	0	3.6 ± 2.5	67.5 ± 13.9	0.556	0.464
		S-D	0	1.5 ± 1.5	56.2 ± 9.4		
	genistein	D-S	0	7.0 ± 7.0	235 ± 54.3	0.397	0.535
		S-D	4.9 ± 4.9	0	282 ± 40.1		
Urine (mg/24 h)	daidzein	D-S	2.0 ± 0.76	2.2 ± 1.4	10.8 ± 1.7	0.300	0.590
		S-D	2.8 ± 1.0	0.28 ± 0.16	14.0 ± 2.5		
	genistein	D-S	0.78 ± 0.29	0.52 ± 0.48	7.6 ± 1.1	3.020	0.097
		S-D	1.5 ± 0.62	0.06 ± 0.06	11.6 ± 2.1		
	Weight (kg)	D-S	75.1 ± 2.5	75.2 ± 2.7	75.4 ± 2.8	0.245	0.626
		S-D	76.8 ± 3.1	77.3 ± 3.4	77.7 ± 3.4		
	BMI (kg/m²)	D-S	25.8 ± 0.88	25.9 ± 1.0	25.9 ± 1.1	0.417	0.525
		S-D	26.6 ± 0.79	26.7 ± 0.91	26.8 ± 0.87		

[†]One-way ANOVA with repeated measures and between groups analysis.

CHAPTER 5

EFFECTS OF PROBIOTIC AND PREBIOTIC FOODS ON SOY ISOFLAVONE BIOAVAILABILITY

Study Context and Collaborations

This study was designed based on some of the results reported in Chapter 3 and Chapter 4 of this thesis. I was responsible for the study design, implementation and co-ordination as well as all isoflavone and lipid profile analysis. This was a collaborative study and also supplied data for an Honours thesis (Maria de los Angeles) that examined isolated LDL cholesterol, Apo-A and particle size in a limited number of plasma samples; these results are not included in this thesis. I will only report on results of sample collection and analysis that I conducted specifically for my thesis.

5.1 INTRODUCTION

Adult gastrointestinal microfloral composition is considered to be relatively stable, however diet can substantially modify the metabolic activity of certain bacterial populations (Hentges, 1980; Parodi, 1999). The essential role of gut microflora in soy isoflavone bioavailability, the influence of background diet on gut microflora and the large inter-individual variation in isoflavone absorption and metabolism were discussed in Chapter 1. Of the microflora, lactobacilli and bifidobacteria are unique in that they are exclusively beneficial to the host (Gibson, 1998) and thus have been termed “probiotic bacteria” (O'Sullivan, 2001). These are both lactic acid bacteria whose main carbohydrate fermentation product is lactate (Hove *et al.*, 1999); they also have high β -glucosidase activity, pertinent to the initial hydrolysis of isoflavones (Turner *et al.*, 2003). Live cultures, most commonly of *Lactobacillus* and *Bifidobacterium* species (O'Sullivan, 2001), can be provided in food matrices, such as fermented milk products to introduce these probiotic bacteria to the gastrointestinal tract (Cummings *et al.*, 1997). After ingestion, these probiotic bacteria have been shown to survive gastric conditions and subsequently colonise the small intestine and colon by adhering to intestinal epithelium (Gomes and Malcata, 1999).

As an alternative to introducing probiotic bacteria through ingestion, prebiotic dietary components can selectively stimulate the growth and/or activity of specific probiotic bacteria already resident in the gut (Gibson, 1999). Most genera of intestinal microflora are saccharolytic and obtain energy by fermentation of dietary carbohydrates that have escaped digestion in the upper intestinal tract (Parodi, 1999; Salminen *et al.*, 1998). Prebiotics are so named on the basis of their ability to promote the growth of lactic acid producing (probiotic) bacteria (Gibson, 1998). As introduced in Chapter 3, resistant starch is a prebiotic carbohydrate that reaches the large bowel undigested, where it is fermented by the gut microflora, stimulating the growth of bifidobacteria (Brown *et al.*, 1999) and increasing β -glucosidase activity (Silvi *et al.*, 1999).

One of the reported beneficial effects of enhancing probiotic gut bacteria is a reduction in the risk of cardiovascular disease through lipid lowering effects. Regular intake of fermented milk containing *Lactobacillus acidophilus* has been shown to reduce the risk of coronary heart disease by 6 - 10% (Kiebling *et al.*, 2002) and a meta-analysis demonstrated reductions in total cholesterol and LDL of 6% and 9%, respectively with intake of a fermented milk product containing probiotic cultures (Agerholm-Larsen *et al.*, 2000a). Similarly, prebiotic consumption has been shown to lower lipid levels, including marked reductions in serum triglycerides (Taylor and Williams, 1998; Topping and Clifton, 2001)

Thus, probiotics containing viable cultures of *Lactobacillus* and *Bifidobacterium* and prebiotics that selectively stimulate their growth in the gastrointestinal tract can potentially modulate the balance of gut microflora to be beneficial to the host and increase levels of β -glucosidase. Consequently, it was hypothesized that intake of probiotic cultures or a prebiotic dietary component may affect soy isoflavone bioavailability and in addition may produce synergistic effects in relation to lipid-lowering. More specifically, it was also hypothesized that probiotic and prebiotic intake could increase or induce equol production, which may have further implications for health benefits (Atkinson *et al.*, 2005). This study was conducted to examine the effects of probiotic or prebiotic consumption on soy isoflavone bioavailability and blood lipids. The effects of dietary combination of soy with either a probiotic-containing product or a prebiotic on isoflavone bioavailability and lipids were compared with the effects of consumption of soy alone.

5.2 METHODS

5.2.1 Subjects

Male and female subjects were recruited in the local media, from databases of subjects who had participated in previous nutritional trials and through advertisements at the University of Wollongong. Subjects were required to be at least 45 years old and mildly hypercholesterolemic (total cholesterol greater than 5.5 mmol/L). Only women who had been both post-menopausal for more than 12 months and not on hormone replacement therapy (HRT) for the previous 6 months were included. Further exclusion criteria included the use of cholesterol-lowering medication, antibiotics or probiotic-containing foods or supplements within 2 months prior to the study, average alcohol intake of greater than 2 standard drinks per day or cigarette smoking. Ethics approval for this study was granted by the University of Wollongong Human Research Ethics Committee (Ethics Number 02/248) and informed, written consent was obtained from subjects prior to commencement.

5.2.2 Study design

This study was designed to investigate the effects of a soy diet, either alone or in combination with a probiotic or prebiotic food, on isoflavone bioavailability and blood lipids. The study used a randomised crossover design with each subject acting as his or her own control. Initially, a general health questionnaire was administered via telephone and subjects were instructed to maintain their background diets throughout the study. After a two-week wash-in period during which soy, yoghurt and any prebiotics or probiotics were excluded from the diet, subjects commenced the study which involved two 5-week dietary periods separated by a 4-week washout. During the diet intervention phases, all subjects consumed soy milk and soy cereal daily, as control or supplemented with a probiotic or prebiotic intervention. Subjects consumed a test soy meal prior to and

at the end of both dietary periods for determination of the effects of each dietary treatment on isoflavone bioavailability following a single, controlled soy intake.

Subjects were allocated to one of four groups (Pro-1, Pro-2, Pre-1, Pre-2) which determined the intervention (probiotic or prebiotic) and the order of crossover (Figure 5.1). To assist with compliance, subjects with a preference for either the probiotic (yoghurt) or prebiotic (bread) study foods were allocated accordingly. Otherwise, allocation to the probiotic or prebiotic intervention was random as was the order of the crossover. The Pro-1 and Pro-2 groups followed the probiotic intervention, for which Pro-1 consumed soy and control yoghurt with no added bacterial cultures (S + YC) for the first 5 weeks then changed to soy and probiotic yoghurt containing live bacteria (S + YP) in the second 5-week dietary period. Pro-2 consumed these study foods in the reverse order for the two 5-week dietary periods. For the prebiotic intervention, Pre-1 consumed soy alone (SC) in the first 5-week period then soy and bread baked with resistant starch (S + RS) in the second 5-week period, and vice versa for Pre-2.

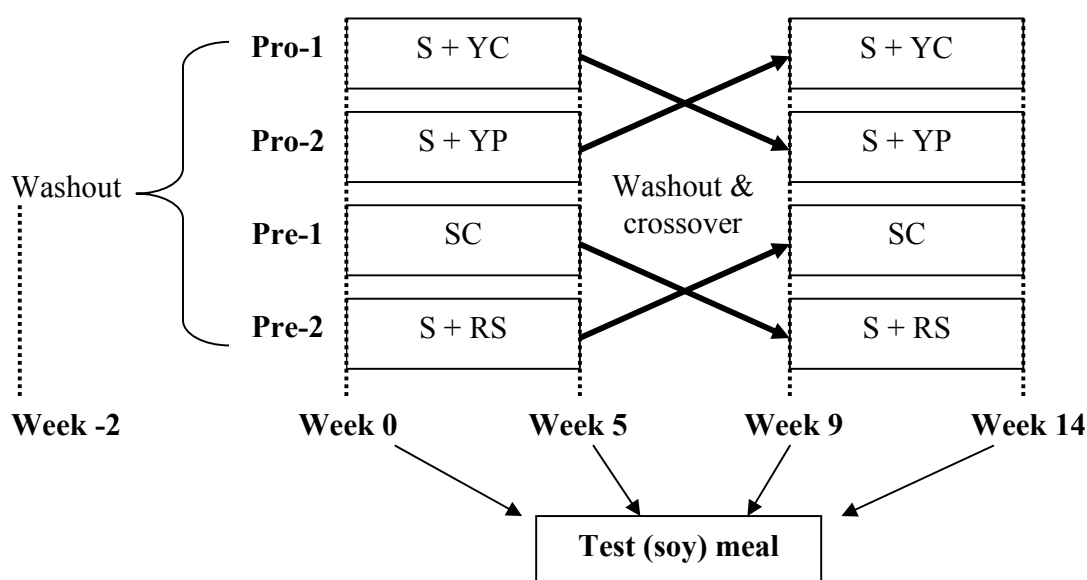


Figure 5.1 Study Design

At the start of weeks 0, 5, 9 and 14 (Figure 5.1), subjects attended the clinic on two consecutive days. On the morning of the first day, height and weight were recorded, a fasted blood sample was taken, a food frequency questionnaire was administered, and subjects commenced a 24 h urine collection. Following the blood sample, a test meal consisting of 250 mL soy milk and 45 g soy cereal was consumed as breakfast in the clinic. Subjects were instructed not to consume any other soy foods, probiotics or prebiotics for 48 h post-meal but otherwise continued their usual activities and diet. They returned to the clinic 8 hours after the test meal for another blood sample. On the following morning, subjects provided a second fasted blood sample and returned their 24 h urine collection. Urine collection continued for the second 24 h period (24 – 48 h) and was returned to the clinic on the 3rd day.

5.2.3 Study foods

All daily foods for both 5-week periods of the study were supplied to subjects from collaborating industry partners. For both 5-week dietary periods, all subjects consumed 250 mL soy milk and 45 g soy cereal daily, identical to those used for the test meals. Soy milk was provided in 1 L cartons, from which subjects were asked to measure and record the volume consumed daily. Soy cereal was pre-packaged as individual 45 g serves. Sufficient quantities of each food for 5 weeks were provided to subjects at the beginning of each dietary period. The test meal consisted of 250 mL of soy milk (So Natural, UHT Calciforte, Taren Point, NSW, Australia) and 45 g soy cereal (Specialty Cereals, Mt Kuring-gai, NSW, Australia) and was consumed at the clinic. The soy cereal was specifically manufactured in a single batch for this study and the soy milk was all from the same lot, thus avoiding between-batch variability in isoflavone levels. The test soy meals and the daily soy intake during the dietary periods provided 38 mg daidzein, 68 mg genistein and 4.5 mg glycitein.

For the probiotic intervention (Pro-1 and Pro-2), subjects consumed an additional 100 mL yoghurt per day during the two 5-week dietary periods. The yoghurts were

manufactured specifically for this study (Vaalia, Pauls Dairy, Brisbane, Qld, Australia) either with (probiotic) or without (control) live bacterial cultures of *Lactobacillus GG*, *Lactobacillus acidophilus* and *Bifidobacterium bifidus*. Both control and probiotic yoghurts were vanilla flavoured with “low” fat (1.4%) content; the probiotic yoghurt contained 10^8 colony forming units (CFU)/100 g daily serve of each of *Lactobacillus acidophilus*, *Bifidobacterium bifidus* and *Lactobacillus GG*. Individual 100 mL serves were pre-packaged in tubs that were unmarked except for a numerical code so that subjects were blind to the type of yoghurt (control or probiotic) they were consuming and there was no difference between them in flavour or texture. Fresh supplies were delivered approximately every 3 weeks and kept refrigerated in the clinic area. Subjects then collected their yoghurt at regular, monitored intervals and maintained their storage under refrigeration.

The prebiotic food was provided to the prebiotic cohort (groups Pre-1 and Pre-2) in the form of 70 % resistant starch flour (Penford Australia Ltd, Sydney, NSW, Australia) that was baked into bread by a local bakery (K & M Bakery, Woonona, NSW, Australia) to provide approximately 4 g resistant starch per slice. Established bread-baking procedures were modified after consultation with Penford Australia Ltd to achieve approximately 4 g RS per slice. Subjects were asked to consume 4 - 5 slices of bread per day (16 - 20 g RS). Bread was baked every 2 - 3 weeks and either delivered to subjects on the day of baking or frozen in the clinic area for pick-up. Subjects were instructed to freeze any bread that they would not consume in the first two days after baking and to thaw as necessary. The low level of water absorption by this resistant starch confers suitability for freezing and thawing (Brown *et al.*, 2000). As there was between batch variation in the exact percent of RS incorporation into the bread and in slice thickness, the number of slices that subjects needed to consume to provide an equivalent of 16 g RS per day varied between 4 and 6, and subjects were advised accordingly.

5.2.4 Sample collection

All blood samples were taken by a registered nurse in the clinic area. Blood was collected into EDTA tubes (Sarstedt) which were inverted and then placed on ice until they were centrifuged at 4°C for 10 min at 3500 rpm. Plasma was harvested and stored in duplicate aliquots at -80°C until analysis. Subjects collected their 24 h urine samples in 2.5 L collection bottles containing 1 g ascorbic acid. The total volume of each 24 h collection was recorded and duplicate 5 mL aliquots stored at -80°C until analysis.

The fasted morning blood sample on the first day of clinic visits was used to assess plasma lipids (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides) and isoflavone concentration (daidzein, genistein, equol, o-desmethylangolensin (ODMA). Isoflavone concentrations in this sample provided an estimate of circulating plasma levels after each dietary period. The blood sample taken 8 h after breakfast was used for analysis of isoflavone concentration. The fasted morning blood sample taken on the second day of clinic visits was used for the repeat determination of plasma lipids, thus taking into account some day-to-day variation and for analysis of isoflavone concentration 24 h after the test meal. Urine samples were analysed for isoflavone concentrations.

5.2.5 Isoflavone quantification in plasma and urine

Isoflavones from plasma and urine were extracted and quantified by HPLC with electrochemical detection as described in 2.3.2 and 2.3.3, respectively.

5.2.6 Lipid analyses

Cholesterol content was determined for total plasma, triglycerides and isolated HDL using standard commercially available kits (Roche Diagnostic) and an autoanalyser

(Cobas Mira Plus). Plasma LDL cholesterol was calculated using the Friedewald calculation (Friedewald *et al.*, 1972).

5.2.7 Statistical analyses

Initially, the normality of data was examined and outliers were identified (SPSS 11.0 and 11.5, SPSS Inc. Chicago, Illinois). ANOVA with repeated measures (ANOVA/RM) and Bonferroni post-hoc analysis (SPSS 11.0 and 11.5, SPSS Inc. Chicago, Illinois) were used wherever possible. Output from ANOVAs were reported with F values, df (hypothesis df, error df) and p values; when presented in Tables, p-values for significant effects ($p < 0.05$) are in bold and p-values for non-significant trends ($0.05 < p < 0.1$) are in italics. When post-hoc tests were not automatically included in the results of ANOVA, Bonferroni correction was used with the significance level adjusted for multiple t-tests. Student's t-tests were 2-tailed (Microsoft Excel, Microsoft Corporation/ SPSS 11.0 and 11.5, SPSS Inc. Chicago, Illinois) and either paired or unpaired depending on the analysis. All values are reported as mean \pm SEM. Correlations are reported as Pearson's Correlation coefficient (R).

5.2.8 Statistical determination of effects of order of dietary interventions

The two cohorts in this study (probiotic and prebiotic) were each made up of two groups that followed a crossover design (Figure 5.1), with a control treatment (S + YC or SC respectively) and an active treatment (S + YP or S + RS respectively). For each cohort, the same treatments for the two groups were compared via two-way ANOVA/RM and between-group analysis to identify whether there were order effects of the dietary treatments. If there were no significant differences between the two groups of a cohort it was concluded that there was no order effect and consequently, the data from the two group were combined.

RESULTS

5.2.9 Subject completion and characteristics

Of 160 people who expressed interest in participating in this study, 70 were eligible for inclusion. After an initial phone questionnaire, 48 people were scheduled to participate, but only 36 (15 females and 21 males) actually commenced the study. Of these, one subject withdrew after the first 5 weeks due to difficulty in blood sampling and intolerance to the soy milk; three others withdrew before the second dietary period due to work or personal commitments; and one subject withdrew during the second dietary period but before the final sample, due to travel commitments. Thus, 31 subjects (12 females and 19 males) completed the study. All females were postmenopausal for at least 12 months, 10 had never used hormone replacement therapy (HRT) and the other two had not in the previous 6 months. Distribution of subjects and mean ages for the four study groups (Pro-1, Pro-2, Pre-1, Pre-2) are presented in Table 5.1. Subject age was not significantly different between the groups ($F = 1.020$, $p = 0.399$, one-way ANOVA with between groups analysis).

Table 5.1 Study groups - subject distribution and dietary intervention order.

Group	Dietary Intervention		n	age at start	
	weeks 0 - 5	weeks 9 - 14		females	males
Pro-1	S + YC	S + YP	10	4	6
Pro-2	S + YP	S + YC	6	2	4
Pre-1	SC	S + RS	9	3	6
Pre-2	S + RS	SC	6	3	3
Total			31	12	19

Neither BMI nor body mass varied significantly across the four test weeks for any of the four groups (Table 5.2) and there were no differences between groups in either body

mass or BMI ($F = 0.097$, $p = 0.961$ and $F = 0.274$, $p = 0.844$, respectively, one-way ANOVA/RM and between groups analysis). However there was a significant overall effect of week on body mass for all subjects and for male subjects (one-way ANOVA/RM, Table 5.2). Although there were no individual significant differences between any of the weeks (Bonferroni post-hoc analysis), the mean body mass of all subjects and of male subjects was highest at week 5. Although males were significantly heavier than females ($F = 9.550$, $p = 0.004$), they did not have a greater BMI ($F = 0.005$, $p = 0.944$, one-way ANOVA/RM and between groups analysis).

Table 5.2 Anthropometric Data. Mean \pm SEM. Body mass (kg); BMI (kg/m^2).

		wk 0	wk 5	wk 9	wk 14	F	df	p [†]
Pro-1	mass	80.0 \pm 4.2	80.5 \pm 4.3	80.1 \pm 4.3	80.0 \pm 4.3	3.288	3,7	0.088
	BMI	27.8 \pm 1.1	27.9 \pm 1.1	28.0 \pm 1.2	28.0 \pm 1.1	0.769	3,7	0.547
Pro-2	mass	82.8 \pm 5.0	83.1 \pm 4.7	82.1 \pm 4.4	81.3 \pm 3.9	1.050	3,3	0.485
	BMI	29.0 \pm 1.2	29.4 \pm 1.1	29.0 \pm 1.1	28.7 \pm 1.0	4.123	3,3	0.138
Pre-1	mass	82.6 \pm 2.3	82.8 \pm 2.4	82.9 \pm 2.5	83.5 \pm 2.3	3.313	3,6	0.099
	BMI	28.9 \pm 1.0	28.9 \pm 1.0	29.0 \pm 1.0	29.2 \pm 1.0	4.023	3,6	0.069
Pre-2	mass	81.4 \pm 4.8	81.9 \pm 4.9	81.5 \pm 4.9	81.7 \pm 4.9	1.901	3,3	0.306
	BMI	28.0 \pm 0.5	28.2 \pm 0.5	28.1 \pm 0.5	28.3 \pm 0.6	3.743	3,3	0.154
Females	mass	74.7 \pm 3.0	74.8 \pm 3.0	74.7 \pm 3.1	74.8 \pm 3.1	0.212	3,9	0.886
	BMI	28.4 \pm 1.0	28.6 \pm 0.97	28.6 \pm 1.0	28.6 \pm 1.0	0.950	3,9	0.457
Males	mass	85.9 \pm 2.3	86.5 \pm 2.3	85.9 \pm 2.2	85.9 \pm 2.1	3.644	3,16	0.036
	BMI	28.4 \pm 0.65	28.5 \pm 0.67	28.5 \pm 0.65	28.5 \pm 0.62	1.456	3,16	0.264
All subjects	mass	81.6 \pm 2.1	81.9 \pm 2.1	81.6 \pm 2.0	81.6 \pm 2.0	3.066	3,28	0.044
	BMI	28.4 \pm 0.54	28.5 \pm 0.54	28.5 \pm 0.56	28.5 \pm 0.54	1.687	3,28	0.192

[†]One-way ANOVA with repeated measures.

5.2.10 Isoflavone levels at baseline (week 0) and after washout (week 9)

Baseline plasma samples taken prior to intake of the test soy meal (0 h) at week 0 were devoid of daidzein and genistein except for three subjects, for whom plasma levels were 10 - 24 ng/mL daidzein and 24 - 32 ng/mL genistein. At week 9 after the 4-week washout, the 0 h plasma samples of 7 subjects contained daidzein or genistein, between 10 and 55 ng/mL daidzein and between 10 and 24 ng/mL genistein. The three subjects who had measurable levels of plasma isoflavones prior to the test soy meal in week 0 also did so in week 9. There were no significant differences between the four groups for plasma isoflavones at week 0 or week 9 (daidzein: $F = 0.298$, $p = 0.826$ and $F = 0.395$, $p = 0.758$ respectively, genistein: $F = 0.504$, $p = 0.683$ and $F = 0.439$, $p = 0.727$ respectively, one-way ANOVA/RM and between groups analysis, Appendix 5.1). There were also no differences between week 0 and week 9 in plasma isoflavone levels for any group (two-way ANOVA/RM, Appendix 5.1). One female subject in group Pre-1 had unusually high levels of plasma isoflavones throughout the study (up to 811 ng/mL daidzein and 1607 ng/mL genistein after 5 weeks S + RS) and was identified as an extreme outlier in statistical analysis, so her plasma data was not included in subsequent statistical analyses.

There were no statistical differences between any of the four groups for week 0 or week 9 urinary concentrations of either daidzein or genistein (daidzein: $F = 1.682$, $p = 0.195$ and $F = 1.073$, $p = 0.378$ respectively, genistein: $F = 1.498$, $p = 0.238$ and $F = 1.969$, $p = 0.143$ respectively, one-way ANOVA/RM and between groups analysis, Appendix 5.1). There were also no significant differences between the week 0 and week 9 urinary isoflavone excretions for any group (two-way ANOVA/RM, Appendix 5.1), however this approached significance for Pre-1, due to higher urinary genistein in week 9. Thus, as the plasma and urinary isoflavone levels were not significantly different between week 0 and week 9, these “baseline” levels, from which effects of the 5-week dietary periods would be determined, were comparable within subjects and between subjects.

5.2.11 Soy supplementation level

The soy foods consumed daily during each 5-week dietary period and for each test soy meal provided a total of 38 mg daidzein and 68 mg genistein and 4.5 mg glycitein (ratio D/G = 0.56), with the majority of this provided by the cereal (29 mg daidzein + 55 mg genistein + 4.5 mg glycitein) and lesser from the milk. This equated to a mean intake of 0.48 ± 0.01 mg daidzein/kg body weight (range 0.37 – 0.77) and 0.84 ± 0.02 mg genistein/kg body weight (range 0.65 – 1.36). The mean intake for females was 0.52 ± 0.03 mg daidzein and 0.92 ± 0.05 mg genistein/kg body weight and for males was 0.45 ± 0.01 mg daidzein and 0.79 ± 0.02 mg genistein/kg body weight.

5.2.12 Effects of probiotic yoghurt on plasma isoflavones after soy intake

There were no overall statistical differences between the two probiotic groups for plasma levels of daidzein or genistein during either the control dietary period (daidzein: $F = 0.522$, $p = 0.482$ and genistein: $F = 0.671$, $p = 0.427$) or the active dietary period (daidzein: $F = 1.252$, $p = 0.282$ and genistein: $F = 1.387$, $p = 0.258$, two way ANOVA/RM and between groups analysis). Hence, the two groups were combined for further analysis. Plasma isoflavone levels during the study for separate probiotic groups are included in Appendix 5.2.

For the probiotic cohort (combined Pro-1 and Pro-2), both 5-week dietary periods (S + YC and S + YP) had a significant effect on plasma daidzein and genistein with a significant interaction between week and time (Table 5.3), due to significant increases in 0 h but not 8 h or 24 h plasma isoflavone levels (Table 5.3, Figure 5.2). However, there were no significant differences between S + YC and S + YP treatments ($F_{1,15} = 0.416$, $p = 0.528$ for daidzein and $F_{1,15} = 0.813$, $p = 0.382$ for genistein, three-way ANOVA/RM). The increase in 0 h showed a trend towards being greater after S + YP compared with S + YC, however this was not significant for either daidzein or genistein ($F_{1,15} = 1.897$, $p = 0.189$ and $F_{1,15} = 1.035$, $p = 0.325$ respectively, two-way ANOVA/RM).

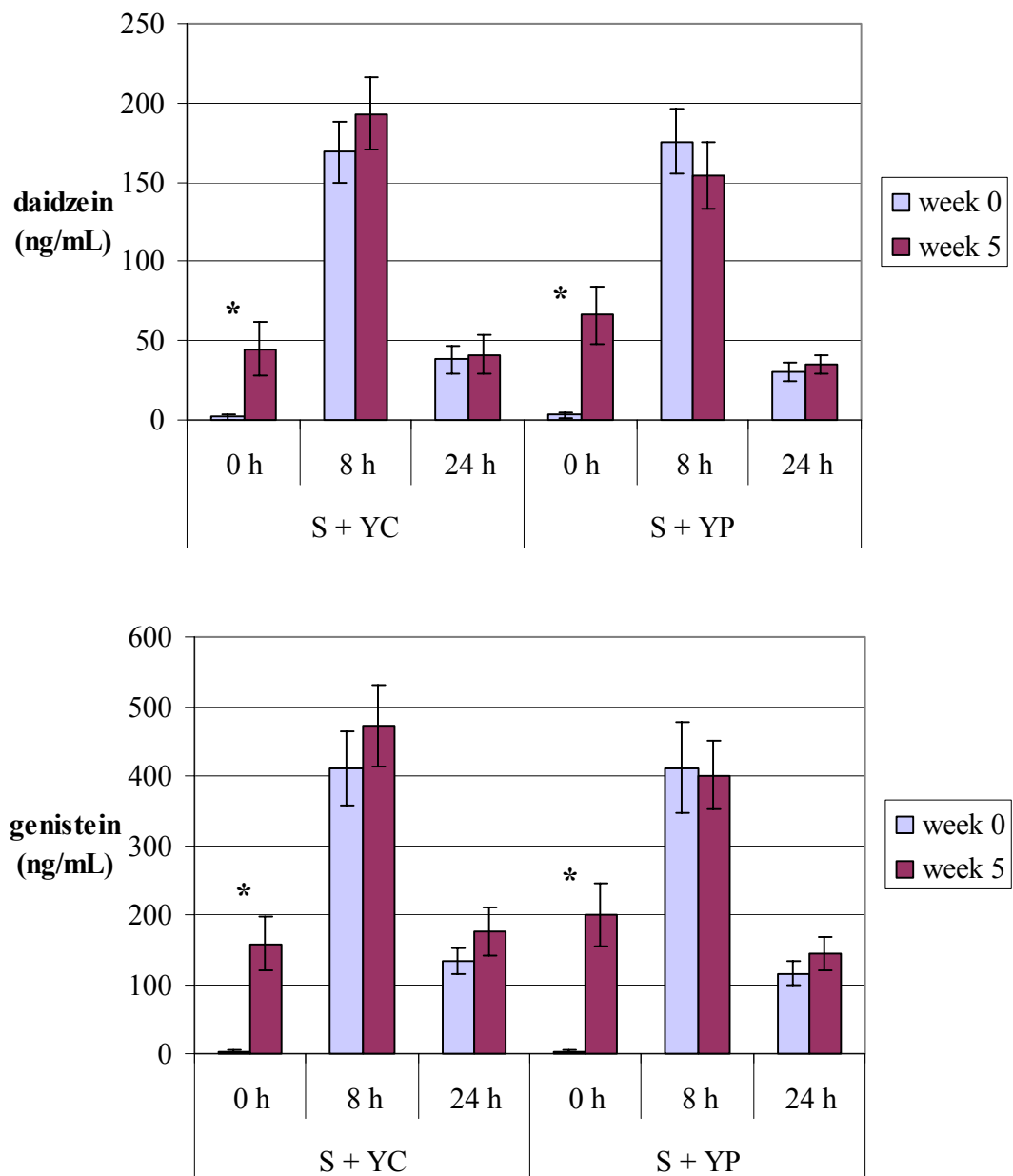


Figure 5.2 Effect of 5 weeks daily intake of soy + control yoghurt (S + YC) and 5 weeks daily intake of soy + probiotic yoghurt (S + YP) on plasma daidzein and genistein for probiotic cohort. Mean ± SEM, n = 16. *Significant $p < 0.017$, post-hoc Student's paired t-tests with Bonferroni correction.

Table 5.3 Probiotic Cohort - Effects of 5 weeks intake of soy + control yoghurt (S + YC) or soy + probiotic yoghurt (S + YP) on plasma daidzein and genistein. Mean \pm SEM (range), n = 16.

			Plasma sample *			time [†]		week [‡]		week x time [‡]	
		Week	0 h	8 h	24 h	F _{2,14}	p	F _{1,15}	p	F _{2,14}	p
daidzein (ng/mL)	S + YC	0	1.9 \pm 1.5 ^a (0 – 23.6)	169 \pm 19.3 ^b (65.3 – 333)	38.3 \pm 8.5 ^c (0 – 156)	36.615	<0.0001	9.905	0.007	5.393	0.018
		5	45.0 \pm 16.8 ^a (0 – 260)	193 \pm 22.7 ^b (50.4 – 350)	41.2 \pm 12.0 ^a (4.1 – 190)	35.195	<0.0001				
	S + YP	0	3.0 \pm 2.2 ^a (0 – 34.2)	176 \pm 20.4 ^b (33.0 – 374)	30.3 \pm 5.7 ^c (0 – 78.5)	33.805	<0.0001	5.636	0.031	5.646	0.016
		5	66.2 \pm 17.8 ^d (4.1 – 237)	154 \pm 20.8 ^e (12.2 – 364)	34.5 \pm 5.9 ^d (0 – 97.0)	17.035	<0.0001				
genistein (ng/mL)	S + YC	0	3.5 \pm 2.4 ^a (0 – 31.9)	412 \pm 53.5 ^b (129 – 1009)	134 \pm 18.0 ^c (23.0 – 281)	34.022	<0.0001	8.205	0.012	5.641	0.061
		5	158 \pm 38.8 ^a (0 – 514)	472 \pm 57.8 ^b (149 – 1019)	175 \pm 34.4 ^a (32.5 – 487)	23.725	<0.0001				
	S + YP	0	2.8 \pm 1.7 ^a (0 – 20.3)	412 \pm 64.7 ^b (67.2 – 972)	116 \pm 17.8 ^c (14.8 – 295)	21.271	<0.0001	13.810	0.002	8.199	0.004
		5	201 \pm 45.5 ^d (46.1 – 682)	401 \pm 49.4 ^e (38.8 – 701)	144 \pm 24.9 ^d (42.4 – 454)	16.387	<0.0001				

* Values in a row with different superscript letters are significantly different. a, b, c - p < 0.002, d – p < 0.05, Bonferroni post-hoc analysis.

† One-way ANOVA with repeated measures.

‡ Two-way ANOVA with repeated measures.

After each test soy meal, plasma isoflavone levels were significantly higher at 8 h than at 0 h and 24 h (Table 5.3). After the test soy meal at the beginning of each dietary period (week 0), daidzein and genistein were still significantly elevated 24 h post-meal compared with 0 h, suggesting that longer than 24 h is needed for a return to baseline levels after soy intake. Mean plasma daidzein and genistein were higher at 0 h of week 5 than at 24 h of week 0; with S + YP this was significant for genistein and almost so for daidzein ($p = 0.043$ and $p = 0.058$, respectively, Student's paired t-tests) but not with S + YC ($p = 0.684$ for daidzein and $p = 0.507$ for genistein, Student's paired t-tests). In addition, while there were no significant differences between 0 h and 24 h plasma isoflavone levels after both dietary periods, after S + YP there was a trend for isoflavone levels to be more elevated at 0 h than 24 h. This suggests that 5 weeks probiotic and soy consumption enhanced circulating plasma isoflavone levels more than the soy control.

When the changes in plasma isoflavone levels were calculated for both 5-week dietary periods (Figure 5.3), there were no significant differences between the two treatments ($F_{1,15} = 0.577$, $p = 0.459$ for daidzein and $F_{1,15} = 0.253$, $p = 0.623$ for genistein, two way ANOVA/RM). However, for both isoflavones there was a significant influence of sample time ($F_{2,14} = 8.681$, $p = 0.004$ and $F_{2,14} = 12.721$, $p = 0.001$ for daidzein and genistein respectively, two-way ANOVA/RM), as there was a significantly greater change in 0 h compared with 24 h levels ($p = 0.003$ and $p = 0.001$, respectively, Bonferroni post-hoc analysis). Although there were no significant overall differences between the two dietary periods, there was a mean increase in 8 h daidzein with S + YC and a mean decrease with S + YP that approached significance ($p = 0.058$, Student's paired t-test with Bonferroni correction of $p = 0.017$).

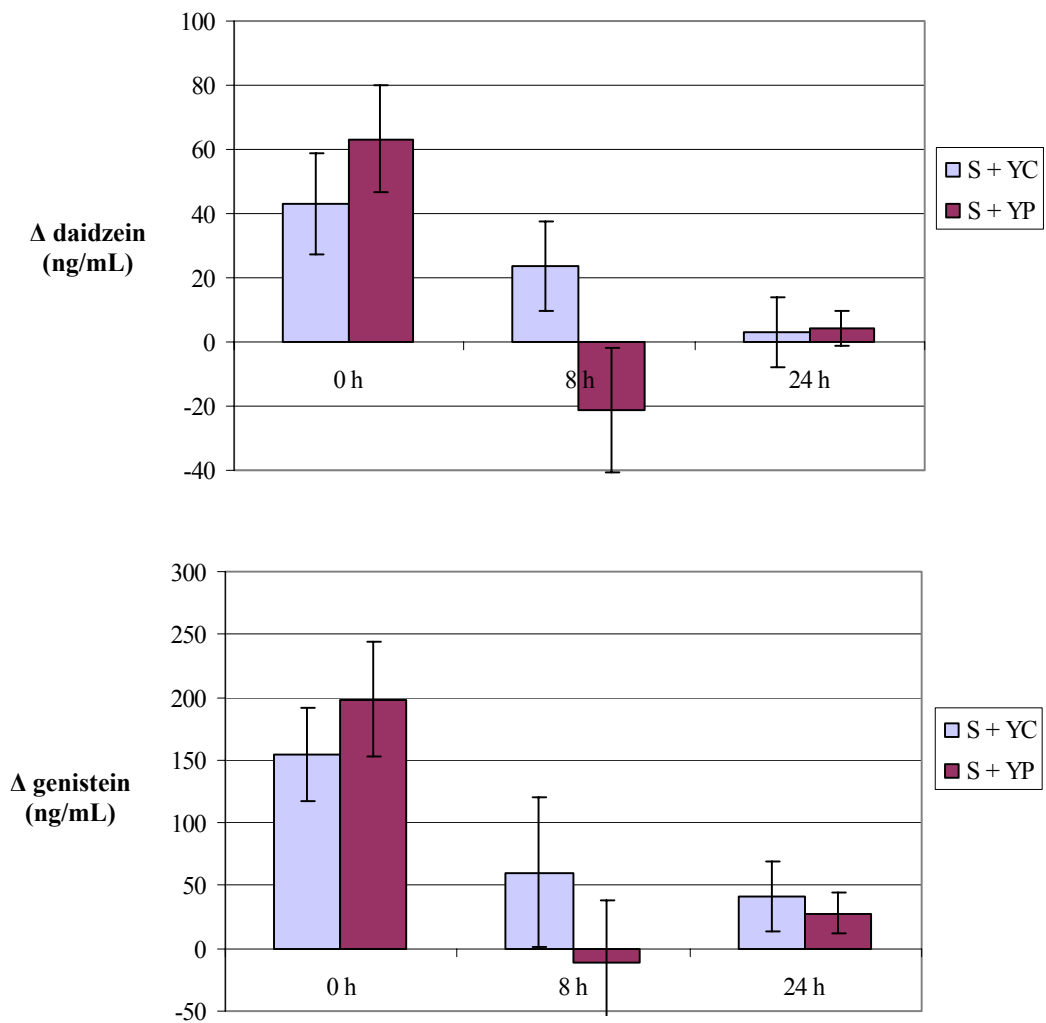


Figure 5.3 Mean absolute change in plasma daidzein and genistein concentrations with 5 weeks soy + control yoghurt (S + YC) and 5 weeks soy + probiotic yoghurt (S + YP). Mean \pm SEM, n = 16.

5.2.13 Probiotic effect on urinary isoflavones

There were no significant differences between the two probiotic groups (S + YC: $F = 0.036$, $p = 0.851$ and $F = 0.755$, $p = 0.400$; S + YP: $F = 0.170$, $p = 0.687$ and $F = 0.029$, $p = 0.866$ for daidzein and genistein, respectively, two way ANOVA/RM and between groups analysis), thus the groups were combined for further analysis. When the two probiotic groups were combined, neither S + YC nor S + YP significantly affected urinary daidzein or genistein concentrations (Table 5.4, Figure 5.4) and there were no significant differences between control and probiotic treatments ($F_{1,15} = 0.013$, $p = 0.909$ and $F_{1,15} = 0.499$, $p = 0.491$ for daidzein and genistein, respectively, three way ANOVA/RM). There was a trend for mean daidzein excretion to have increased more with S + YP than with S + YC. At all weeks, 0 – 24 h urinary isoflavone excretion was significantly higher than 24 – 48 h (Table 5.4). Urinary daidzein and genistein concentrations for separate probiotic groups are included in Appendix 5.3.

Table 5.4 Urinary daidzein and genistein for probiotic cohort with soy + control yoghurt (S + YC) and soy + probiotic yoghurt (S + YP) treatments. Mean \pm SEM (range), n = 16.

			Urine sample			ANOVA [†]		
Wk			0 – 24 h	24 - 48 h	0 – 48 h	F _{1,15}	p	p [‡]
daidzein (mg)	S + YC	0	12.0 ± 1.0 (5.8 – 21.7)	5.0 ± 1.4 (0.79 – 21.6)	17.0 ± 1.9 (10.1 – 37.6)	0.128	0.726	0.001
		5	13.2 ± 1.3 (3.2 – 20.4)	4.6 ± 1.4 (0.47 – 23.1)	17.8 ± 2.1 (4.0 – 38.1)			<0.0001
	S + YP	0	12.0 ± 1.3 (3.8 – 20.1)	4.1 ± 1.1 (0.68 – 16.8)	16.1 ± 2.0 (5.8 – 30.4)	1.576	0.229	<0.0001
		5	14.2 ± 1.6 (4.0 – 27.8)	4.8 ± 0.91 (1.6 – 15.5)	19.0 ± 2.1 (5.6 – 34.5)			<0.0001
genistein (mg)	S + YC	0	6.1 ± 0.68 (2.1 – 11.8)	2.4 ± 0.80 (0 – 11.8)	8.5 ± 1.1 (2.5 – 16.0)	1.242	0.283	0.003
		5	7.6 ± 1.0 (1.5 – 17.1)	3.1 ± 1.5 (0 – 25.3)	10.8 ± 2.2 (2.7 – 38.3)			0.006
	S + YP	0	5.8 ± 0.79 (1.3 – 10.9)	2.0 ± 0.91 (0 – 15.1)	7.7 ± 1.3 (1.6 – 20.6)	2.424	0.140	0.005
		5	7.4 ± 0.97 (0.81 – 16.9)	2.5 ± 0.62 (0 – 7.4)	9.9 ± 1.5 (0.81 – 23.9)			<0.0001

[†]Two way ANOVA with repeated measures for effect of dietary period on 0 – 24 h and 24 – 48 h urinary isoflavone excretion.

[‡]Student's paired t-tests for comparison between 0 – 24 h and 24 – 48 h.

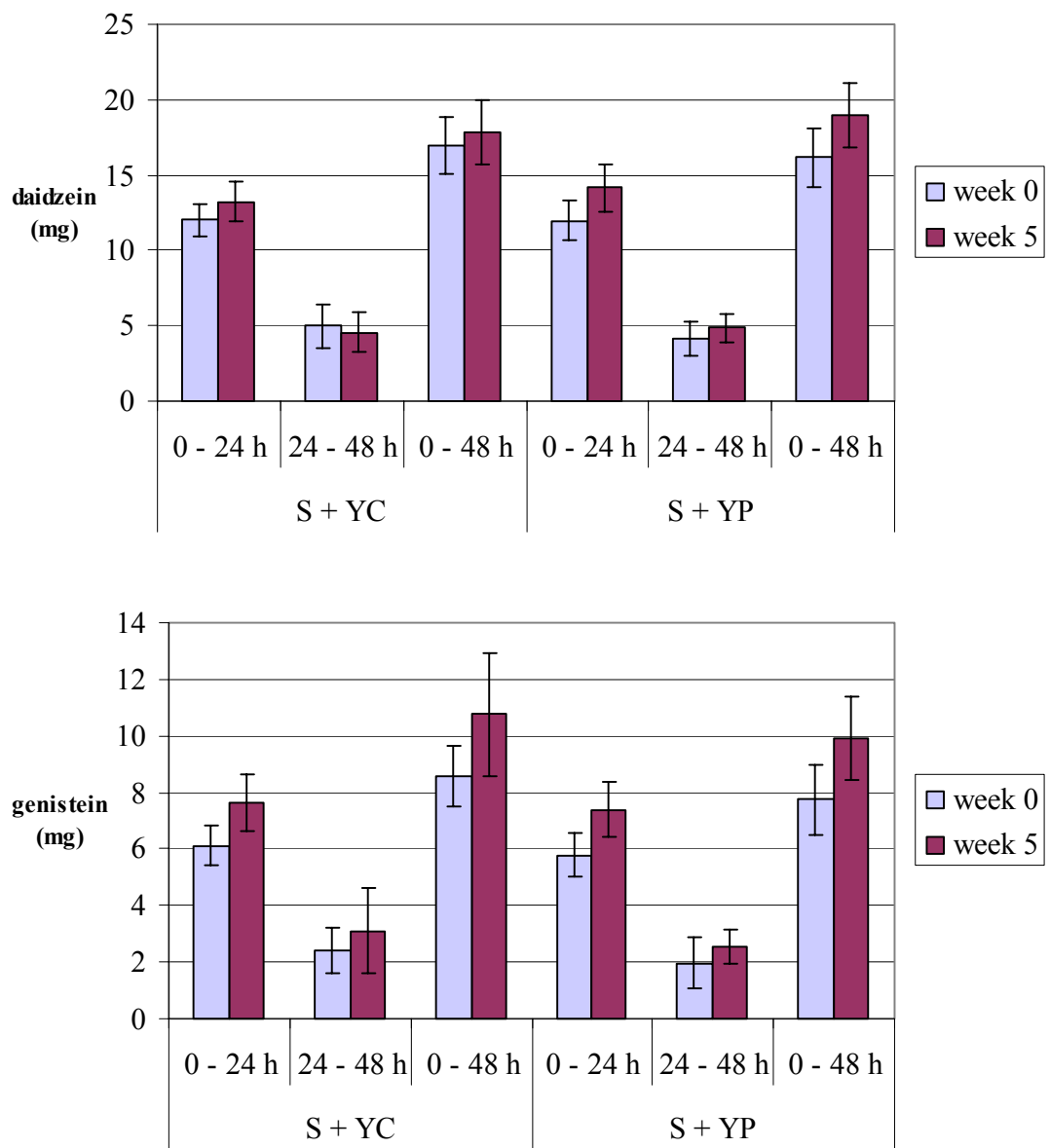


Figure 5.4 Urinary daidzein and genistein excretion before and after 5 weeks of soy + control yoghurt (S + YC) and 5 weeks of soy + probiotic yoghurt (S + YP). Mean \pm SEM, n = 16.

When the changes in urinary isoflavone excretion over the 5-week dietary periods were compared, there were no significant differences between S + YC and S + YP treatments ($F_{1,15} = 0.472$, $p = 0.503$ for daidzein and $F_{1,15} = 0.000$, $p = 0.984$ for genistein, two way ANOVA/RM, Figure 5.5), although there was a trend for greater total daidzein excretion with the probiotic treatment. Both treatments resulted in a greater mean increase in 0 – 24 h urinary excretion of daidzein and genistein compared with 24 – 48 h, but these were not significant ($F_{1,15} = 1.508$, $p = 0.238$ for daidzein and $F_{1,15} = 0.814$, $p = 0.381$ for genistein, two way ANOVA/RM).

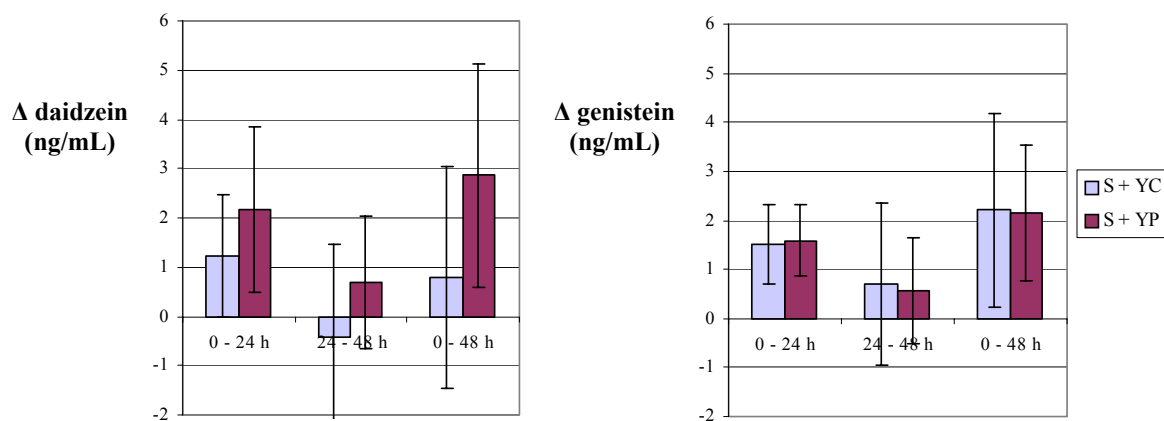


Figure 5.5 Mean absolute change in urinary daidzein and genistein excretion with 5 weeks soy + control yoghurt (S + YC) and 5 weeks soy + probiotic yoghurt (S + YP). Mean \pm SEM, $n = 16$.

To account for some of the large inter-individual variability, changes were expressed as percent change. Again, there were no differences between the treatments ($F_{1,15} = 0.001$, $p = 0.971$ for daidzein and $F_{1,15} = 0.513$, $p = 0.489$ for genistein, two way ANOVA/RM, Figure 5.6), although there was a trend towards greater mean excretion of both isoflavones with S + YP. All the mean percent changes were positive, and tended to be greater for urinary isoflavone excretion between 24 – 48 h compared with 0 – 24 h; this approached significance for genistein but was not significant for daidzein ($F_{1,15} = 4.153$, $p = 0.066$ and $F_{1,15} = 1.834$, $p = 0.196$, respectively, two-way ANOVA/RM).

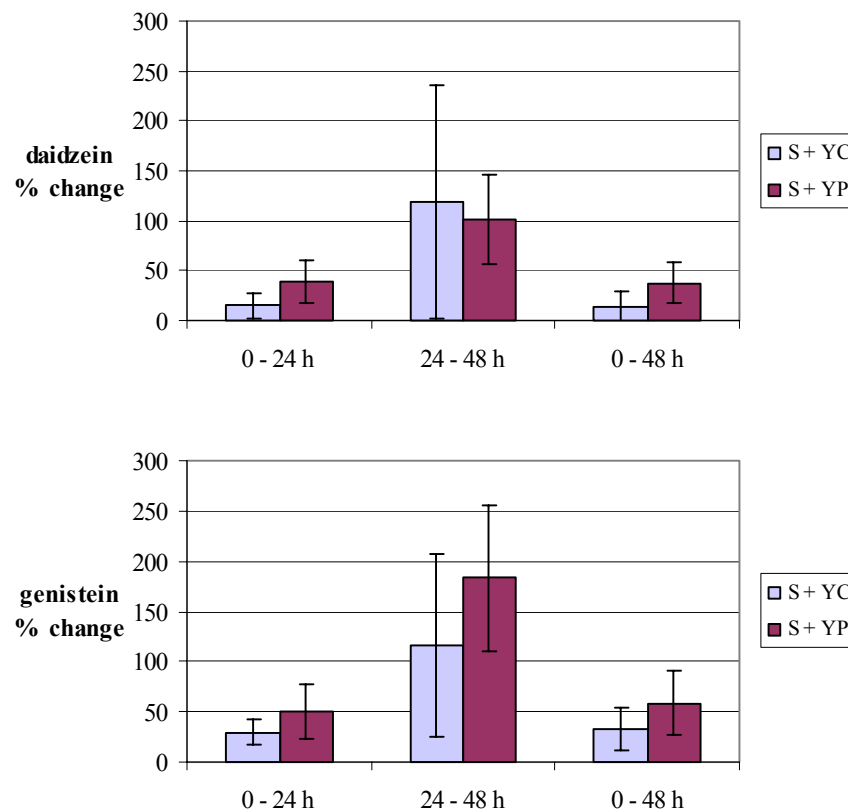


Figure 5.6 Mean percent change in urinary daidzein and genistein excretion with 5 weeks of soy + control yoghurt (S + YC) and 5 weeks soy + probiotic yoghurt (S + YP). Mean \pm SEM, n = 16.

5.2.14 Summary of Probiotic Effects on Plasma and Urinary Isoflavones

Overall, there were no significant differences between the effects of 5 weeks daily intake of soy + probiotic yoghurt compared with 5 weeks daily intake of soy + control yoghurt on plasma or urinary isoflavone levels. While both 5-week dietary periods significantly increased plasma daidzein and genistein, this was due to increases only in the 0 h samples, which had minimal isoflavone concentrations prior to soy intake for both treatments. Urinary isoflavone excretion was not significantly affected by either

treatment and there were no differences between treatments, but there was a trend for urinary excretion of daidzein and genistein to increase with both 5-week dietary periods. There was large variability between subjects in their plasma and urinary concentrations which may have masked the significance of some results. Overall, there appeared to be some evidence for increased plasma levels of isoflavones and a small percent increase in urinary isoflavone excretion after 5 weeks of daily soy intake compared with after a single soy meal. While there were no significant additional effects of probiotic intake with soy, there was a trend for greater increases in 0 h plasma daidzein and genistein and urinary daidzein excretion with probiotic and soy intake than with the soy control.

5.2.15 Prebiotic effects of RS intake on plasma isoflavones

There were no statistical differences between the two prebiotic groups for plasma daidzein or genistein with either SC or S + RS (daidzein: $F = 0.002$, $p = 0.967$ and $F = 0.820$, $p = 0.384$, respectively, genistein: $F = 0.020$, $p = 0.889$ and $F = 1.310$, $p = 0.277$, respectively, two-way ANOVA/RM and between groups analysis). The groups were thus combined for further analysis. For the combined prebiotic cohort, there was a significant effect of both 5-week dietary periods (SC and S + RS) on plasma daidzein and genistein levels. However, there were interactions between week and time (Table 5.5) as not all samples were significantly increased (Figure 5.7). There were no significant differences between the two treatments (SC and S + RS) for either plasma daidzein or genistein ($F_{1,15} = 0.416$, $p = 0.528$ and $F_{1,15} = 0.813$, $p = 0.382$ respectively, three-way ANOVA/RM). Plasma isoflavone concentrations during the study for separate prebiotic groups are included in Appendix 5.4

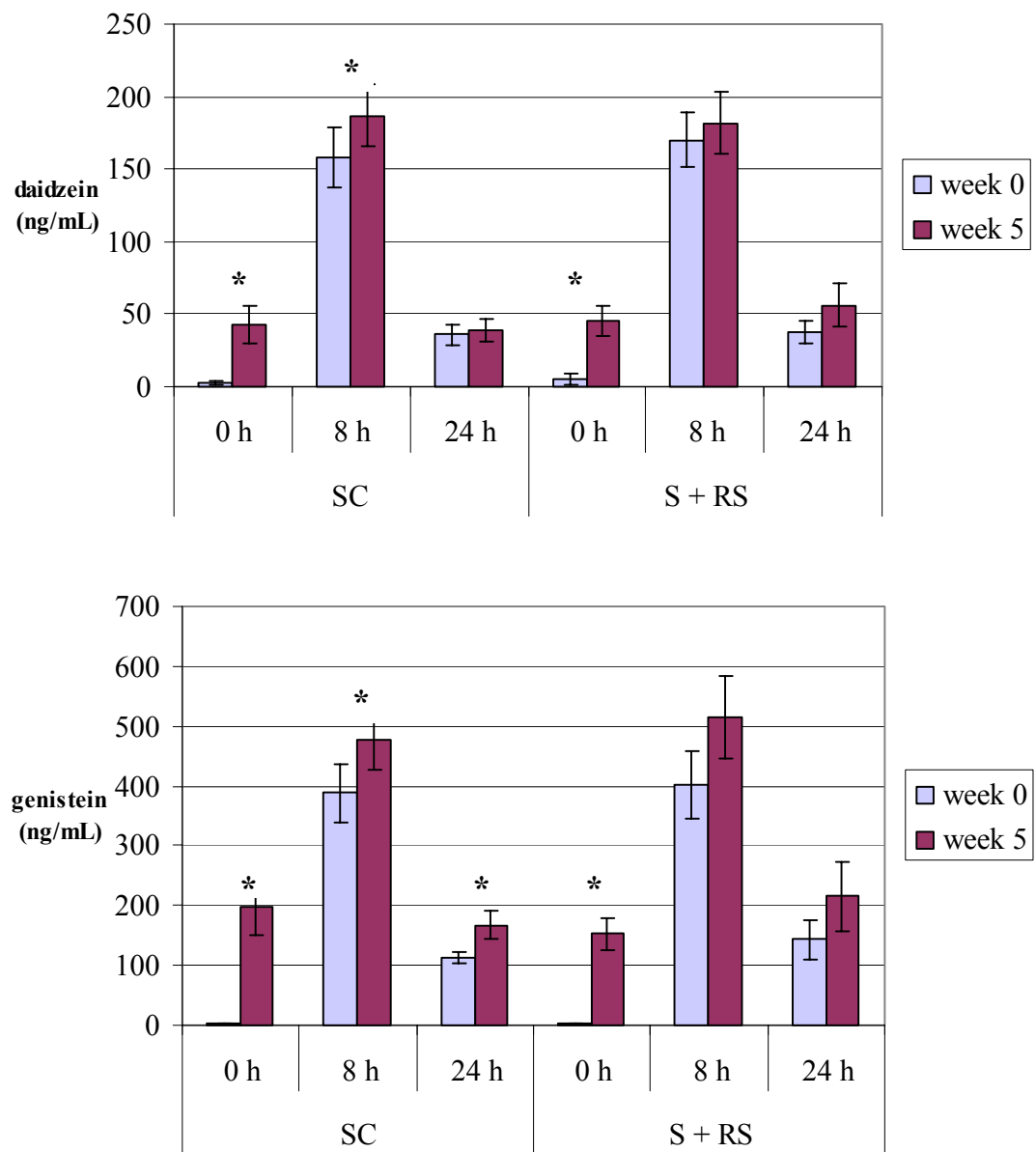


Figure 5.7 Prebiotic cohort - effect of 5 weeks soy control (SC) and 5 weeks soy + resistant starch (S + RS) on mean plasma daidzein and genistein levels. Mean \pm SEM, n = 14. *Significant $p < 0.017$, post-hoc Student's paired t-tests with Bonferroni correction.

Table 5.5 Prebiotic Cohort - Effects of 5 weeks intake of soy control (SC) or soy + resistant starch (S + RS) on plasma daidzein and genistein. Mean \pm SEM (range), n = 14.

			Plasma sample *			time [†]		week [‡]		week x time [‡]	
Week			0 h	8 h	24 h	F _{2,11}	p	F _{1,12}	p	F _{2,11}	p
daidzein (ng/mL)	SC	0	2.2 ± 1.2 ^a (0 – 13.1)	158 ± 20.4 ^b (72.9 – 303)	36.0 ± 7.6 ^c (9.1 – 104)	25.893	<0.0001	18.710	0.001	5.401	0.023
		5	43.1 ± 12.9 ^a (0 – 162)	187 ± 21.7 ^b (57.1 – 346)	38.7 ± 7.9 ^a (6.1 – 104)	47.316	<0.0001				
	S + RS	0	4.9 ± 3.9 ^a (0 – 54.7)	170 ± 19.0 ^b (68.3 – 324)	37.5 ± 8.1 ^c (8.4 – 93.4)	39.946	<0.0001	5.355	0.039	4.569	0.036
		5	45.7 ± 10.4 ^a (0 – 133)	182 ± 21.1 ^b (66.0 – 357)	56.3 ± 15.1 ^a (12.3 – 198)	44.655	<0.0001				
genistein (ng/mL)	SC	0	1.7 ± 1.2 ^a (0 – 14.7)	388 ± 49.2 ^b (167 – 744)	113 ± 10.6 ^c (61.8 – 184)	49.971	<0.0001	45.825	0.000	4.900	0.030
		5	197 ± 47.4 ^a (58.3 – 598)	479 ± 51.6 ^b (139 – 800)	167 ± 24.0 ^a (66.6 – 365)	51.883	<0.0001				
	S + RS	0	2.2 ± 1.7 ^a (0 – 24.1)	403 ± 56.3 ^b (129 – 938)	143 ± 33.7 ^c (44.6 – 486)	52.852	<0.0001	14.044	0.003	4.484	0.038
		5	153 ± 27.7 ^a (0 – 355)	516 ± 69.1 ^b (140 – 1010)	215 ± 60.2 ^a (67.5 – 876)	30.018	<0.0001				

* Values in a row with different superscript letters are significantly different. p < 0.02, Bonferroni post-hoc analysis.

[†]One-way ANOVA with repeated measures.

[‡]Two-way ANOVA with repeated measures.

During weeks 0 and 5 of both dietary periods, plasma daidzein and genistein were significantly elevated 8 h post-meal compared with 0 h and 24 h (Table 5.5). At week 0, plasma daidzein and genistein were significantly greater at 24 h than at 0 h, but after both 5-week dietary periods, there were no significant differences between 0 h and 24 h levels due to elevated levels at 0 h. There was a trend for the mean 0 h levels at week 5 to be higher than the mean 24 h levels for week 0 of the corresponding dietary period, suggesting increased plasma accumulation with daily soy intake. There was also a trend for the 24 h plasma levels to have increased after S + RS.

For the change in plasma daidzein and genistein with both 5-week dietary periods, there were no differences between SC and S + RS treatments ($F_{1,12} = 0.043$, $p = 0.840$ for daidzein and $F_{1,12} = 0.011$, $p = 0.919$ for genistein, two way ANOVA/RM). However, there was a significant effect of sample time for both daidzein and genistein ($F_{2,11} = 8.536$, $p = 0.006$ and $F_{2,11} = 7.121$, $p = 0.010$, respectively, two-way ANOVA/RM), as there was a significantly greater increase in 0 h daidzein and genistein levels compared with 24 h levels ($p = 0.003$ and $p = 0.016$, respectively, Bonferroni post-hoc analysis).

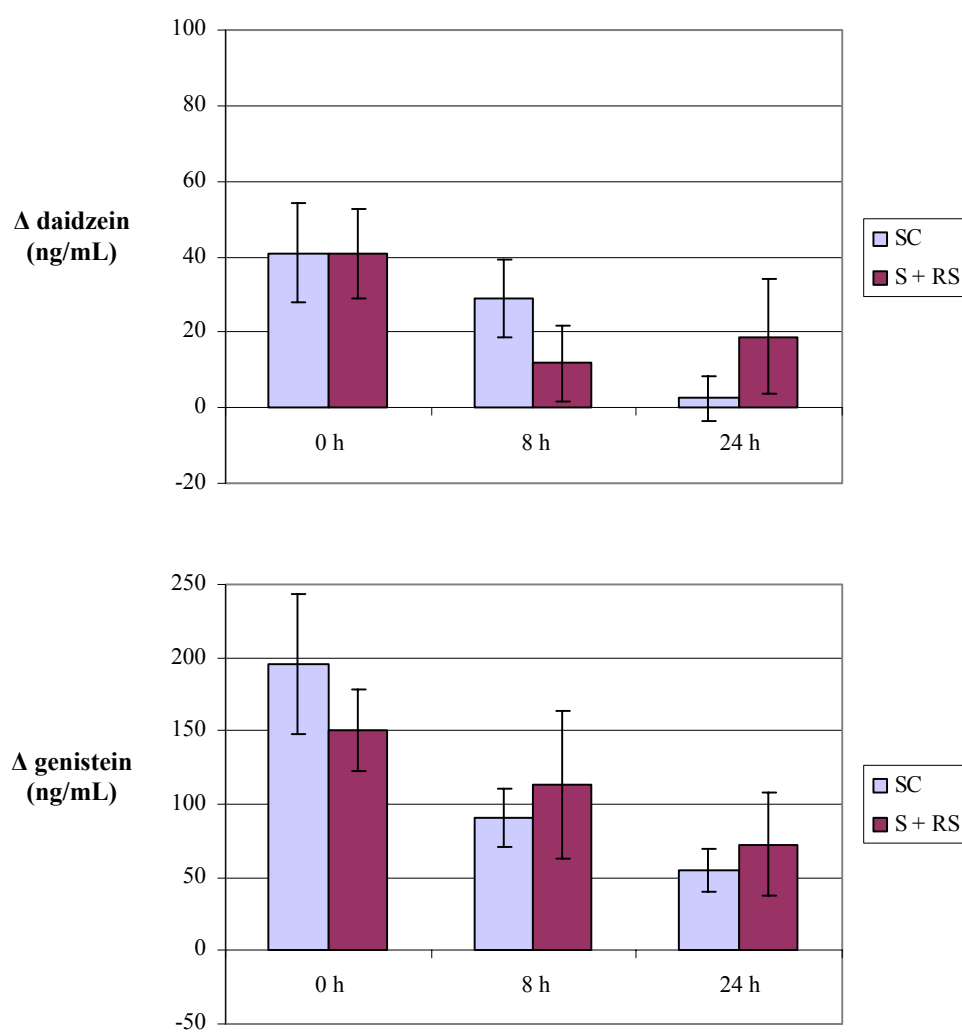


Figure 5.8 Mean absolute change in plasma daidzein and genistein levels after 5 weeks soy control (SC) or 5 weeks soy + resistant starch (S + RS) for prebiotic cohort. Mean \pm SEM, n = 14.

5.2.16 Prebiotic Effect on Urinary Isoflavones

Although there were no significant differences between the two prebiotic groups for either treatment (SC: $F = 0.646$, $p = 0.437$ for daidzein and $F = 0.394$, $p = 0.542$ for genistein; S + RS: $F = 3.066$, $p = 0.105$ for daidzein and $F = 2.240$, $p = 0.160$ for genistein, two way ANOVA/RM), enabling groups to be combined, it should be noted that Pre-2 had unusually high urinary daidzein and genistein levels at the beginning of S + RS treatment which may have masked the effects of this treatment. When the two probiotic groups were combined, there were no significant differences between the SC and S + RS treatments for urinary isoflavone excretion ($F_{1,13} = 0.537$, $p = 0.477$ for daidzein and $F_{1,13} = 3.287$, $p = 0.093$ for genistein, three way ANOVA/RM). Neither of the treatments affected urinary daidzein or genistein excretion, although there was a trend for increased genistein excretion after 5 weeks of soy control that approached significance (Table 5.6). At all weeks, the 0 – 24 h urinary excretion of both daidzein and genistein was significantly higher than that between 24 – 48 h (Table 5.6). Urinary isoflavone levels for separate prebiotic groups are included in Appendix 5.5.

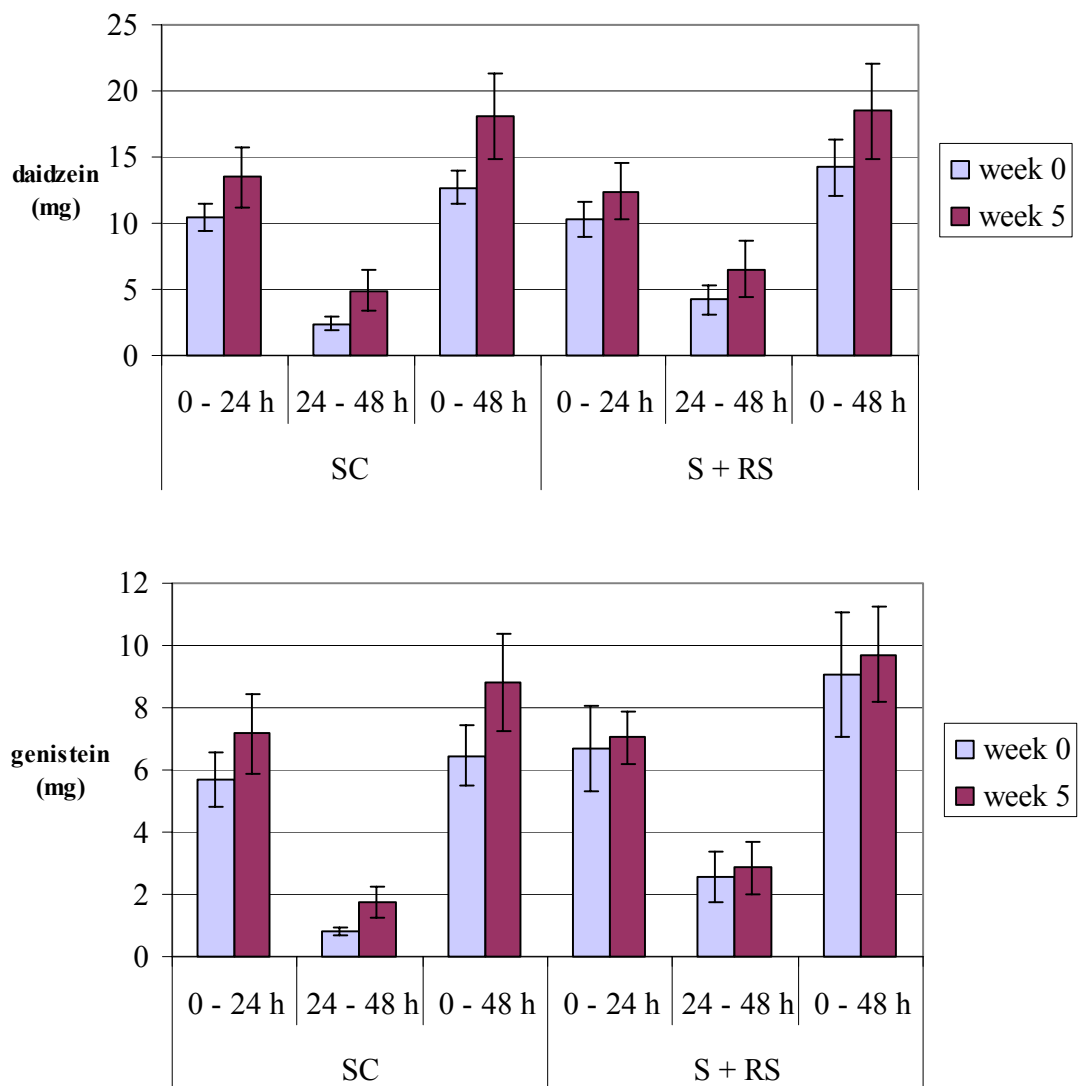


Figure 5.9 Mean urinary excretion of daidzein and genistein with 5 weeks soy control (SC) or 5 weeks soy + resistant starch (S + RS) for prebiotic cohort. Mean \pm SEM, n = 14.

Table 5.6 Urinary daidzein and genistein for prebiotic cohort at weeks 0 and 5 of soy control and soy + resistant starch (S + RS) prebiotic treatments. Mean \pm SEM (range), n = 14.

			Urine sample			ANOVA [†]		p [‡]
		Wk	0 – 24 h	24 – 48 h	0 – 48 h	F _{1,13}	p	
daidzein (mg)	SC	0	10.4 \pm 2.4 (2.8 – 18.3)	2.4 \pm 0.45 (0.57 – 18.3)	12.7 \pm 1.2 (3.3 – 21.4)	2.650	0.128	<0.0001
		5	13.5 \pm 2.3 (0 – 34.8)	4.9 \pm 1.6 (0.69 – 23.0)	18.1 \pm 3.2 (2.0 – 57.9)			0.001
	S + RS	0	10.3 \pm 1.4 (3.3 – 21.3)	4.2 \pm 1.1 (0.80 – 13.9)	14.2 \pm 2.2 (4.3 – 28.9)	0.689	0.421	<0.0001
		5	12.4 \pm 2.1 (4.2 – 39.0)	6.5 \pm 2.1 (1.2 – 25.7)	18.5 \pm 3.6 (5.9 – 61.3)			0.013
	SC	0	5.7 \pm 0.87 (1.4 – 13.6)	0.81 \pm 0.14 (0.20 – 2.2)	6.4 \pm 0.97 (1.6 – 15.8)	3.781	0.074	<0.0001
		5	7.2 \pm 1.3 (0 – 16.7)	1.8 \pm 0.52 (0 – 6.2)	8.8 \pm 1.6 (0.60 – 22.9)			0.001
genistein (mg)	S + RS	0	6.7 \pm 1.4 (1.5 – 17.4)	2.6 \pm 0.82 (0 – 9.1)	9.1 \pm 2.0 (1.5 – 25.2)	0.036	0.853	0.001
		5	7.0 \pm 0.84 (2.3 – 13.4)	2.8 \pm 0.86 (0.30 – 10.5)	9.7 \pm 1.5 (2.6 – 21.9)			<0.0001

[†]Two way ANOVA with repeated measures for effect of dietary period on 0 – 24 h and 24 – 48 h urinary isoflavone excretion.

[‡]Student's paired t-tests for comparison between 0 – 24 h and 24 – 48 h.

There were no significant differences between treatments for the change in urinary isoflavone excretion after each 5-week period ($F_{1,13} = 0.012$, $p = 0.916$ and $F_{1,13} = 0.470$, $p = 0.505$ respectively, two way ANOVA/RM), but variability was high (Figure 5.10). There were also no differences between the samples (0 – 24 h and 24 – 48 h) in the effect of either dietary period ($F_{1,13} = 0.067$, $p = 0.799$ for daidzein and $F_{1,13} = 0.672$, $p = 0.427$ for genistein, two way ANOVA/RM).

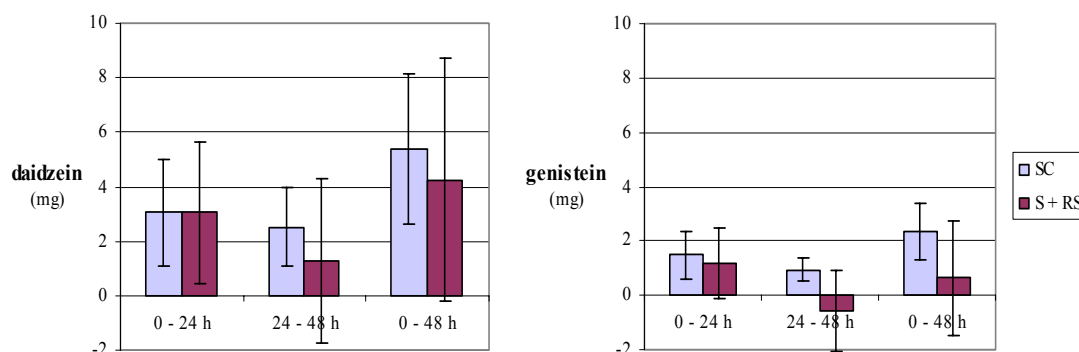


Figure 5.10 Mean absolute change in urinary daidzein and genistein excretion with soy control (SC) and soy + resistant starch (S + RS) treatments for prebiotic cohort. Mean \pm SEM, $n = 14$.

When expressed as percent changes for both 5-week dietary periods, there were still no significant differences between the treatments ($F_{1,13} = 0.389$, $p = 0.544$ for daidzein and $F_{1,12} = 1.571$, $p = 0.234$ for genistein, two way ANOVA with repeated measures), although there was a trend for greater mean excretion of both isoflavones with prebiotic treatment compared with control. All percent changes were positive and of greater magnitude for urinary excretion 24 – 48 h compared with 0 – 24 h, this was significant for daidzein and approached significance for genistein ($F_{1,13} = 5.720$, $p = 0.033$ for daidzein and $F_{1,12} = 3.682$, $p = 0.079$ for genistein, two way ANOVA/RM).

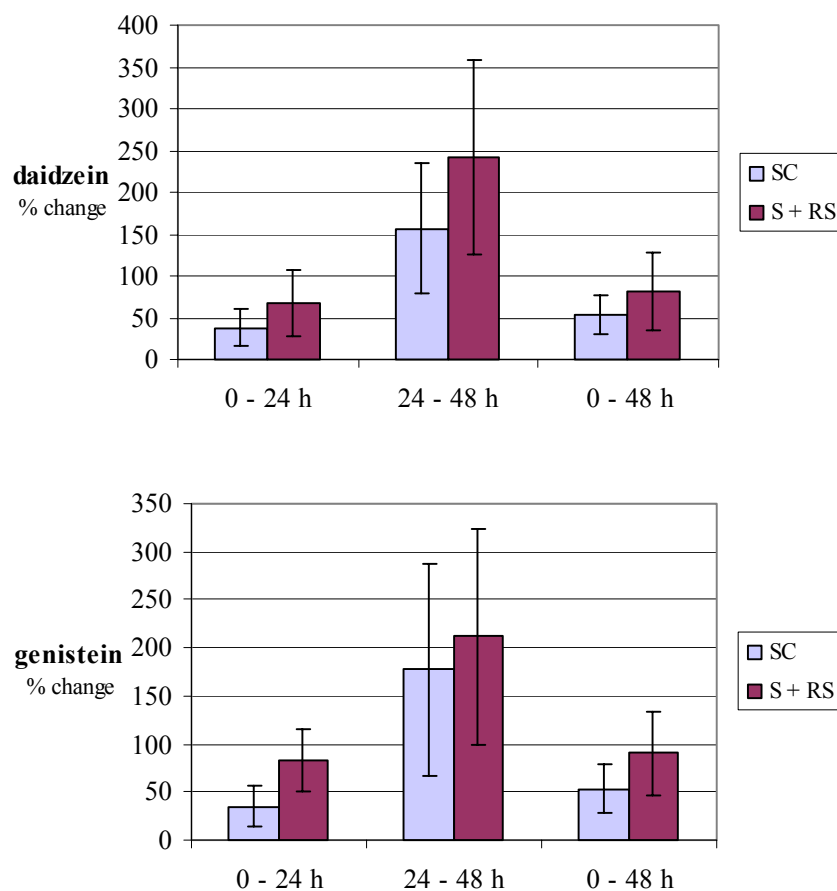


Figure 5.11 Mean percent change of urinary daidzein and genistein excretion with soy control (SC) and soy + resistant starch (S + RS) treatments. Mean \pm SEM, n = 14

5.2.17 Summary of prebiotic effects on plasma and urinary isoflavones

For the prebiotic cohort, there were no significant differences between the effects of control and prebiotic treatments on plasma or urinary isoflavones. Plasma daidzein and genistein were significantly increased at 0 h after both SC and S + RS and there was a trend for higher 24 h plasma isoflavone levels after 5 weeks daily intake of soy. The 24 h plasma levels of daidzein and genistein were both the highest after 5 weeks of soy and

resistant starch consumption, suggesting that this treatment may have enhanced isoflavone uptake from the single test soy meal.

5.2.18 Comparison between probiotic and prebiotic effects

When the active treatments for the probiotic and prebiotic cohorts (S + YP and S + RS respectively) were compared, the effect on plasma isoflavones was not significantly different between S + YP and S + RS ($F = 0.440$, $p = 0.513$ for daidzein and $F = 0.623$, $p = 0.437$ for genistein, two-way ANOVA/RM and between-groups analysis). There was a trend for a greater mean increase in 0 h plasma daidzein and genistein with S + YP and for a greater mean increase in 8 h and 24 h plasma daidzein and genistein with S + RS (Figure 5.12). Although none of these trends were significant, the mean change in 8 h isoflavone plasma levels showed the greatest difference between treatments. The ratio of daidzein: genistein in plasma was also not significantly different between the active treatments of the two cohorts ($F = 0.004$, $p = 0.952$, two-way ANOVA/RM and between groups analysis, Table 5.7).

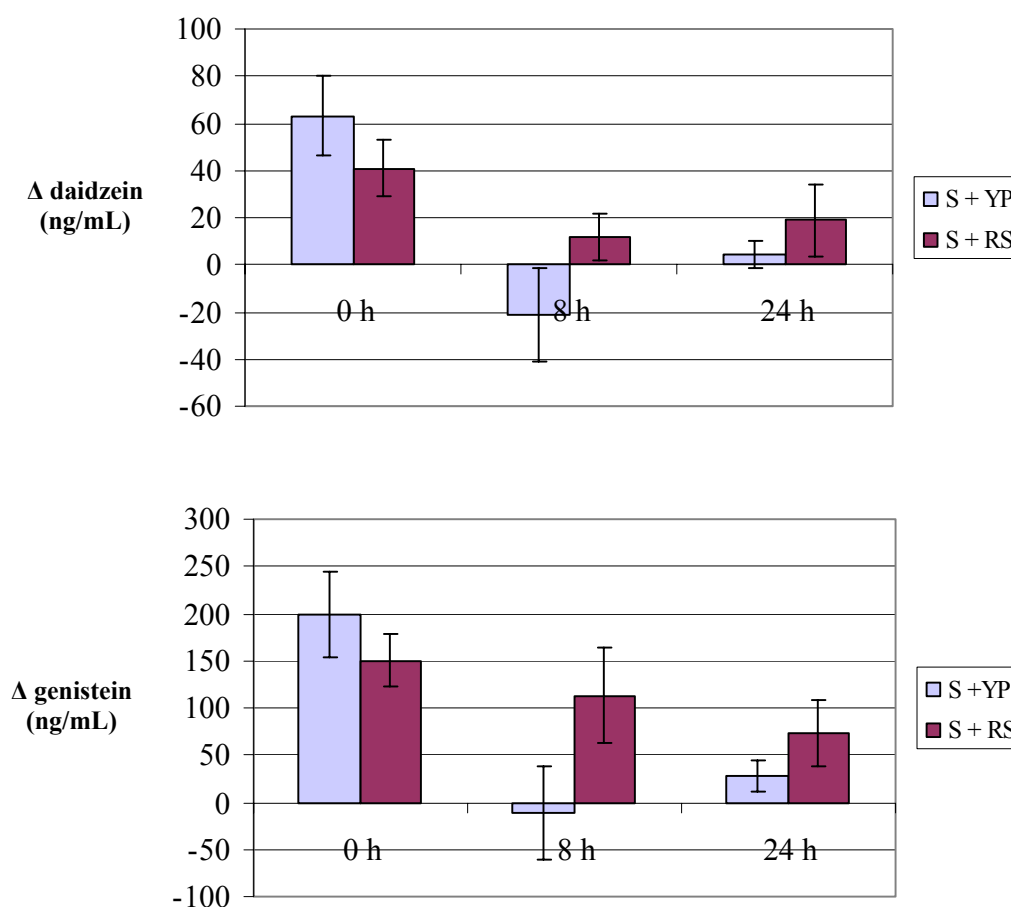


Figure 5.12 Comparison between probiotic and prebiotic cohorts - mean change in plasma isoflavone concentrations. Mean \pm SEM, Pro: n = 16, Pre: n = 14.

The urinary excretions of both daidzein and genistein were also very similar between the cohorts with no significant differences between probiotic and prebiotic treatments ($F = 0.066$, $p = 0.800$ for daidzein and $F = 0.256$, $p = 0.617$, two-way ANOVA with between groups analysis). The mean total 48 h urinary excretion of daidzein increased from 16.1 ± 2.0 to 19.0 ± 2.1 mg (18%) with probiotic treatment and from 14.2 ± 2.2 to 18.5 ± 3.6 mg (30%) with prebiotic treatment, while urinary genistein excretion increased from 7.7 ± 1.3 to 9.9 ± 1.5 mg (29%) with probiotic treatment and from 9.1 ± 2.0 to 9.7 ± 1.5 mg (7%) with prebiotic treatment. Thus, there was a trend for a greater increase in urinary

genistein excretion with probiotic treatment and for a greater increase in urinary daidzein excretion with prebiotic treatment. However, when the percent changes in urinary excretion were compared, there were no significant differences between probiotic and prebiotic cohorts ($F = 1.315$, $p = 0.261$ for daidzein and $F = 0.015$, $p = 0.902$ for genistein, two-way ANOVA/RM and between-groups analysis). Although there was high variability, there was a trend suggesting greater mean percent increase in urinary isoflavone excretion with the prebiotic cohort, particularly in the urinary daidzein content of the 24 – 48 h sample period (Figure 5.13). There were also no differences between probiotic and prebiotic treatments in the urinary daidzein: genistein ratio over the 5 weeks ($F < 0.0001$, $p = 0.997$, three-way ANOVA/RM and between groups analysis; Table 5.7).

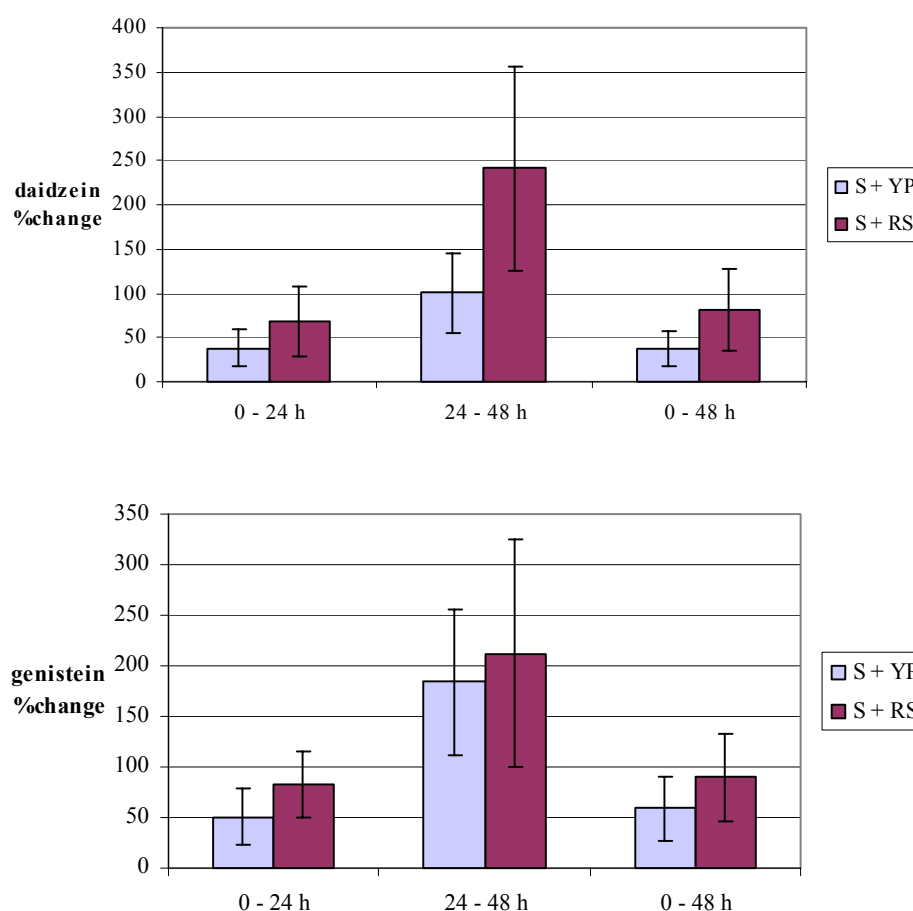


Figure 5.13 Mean changes in urinary isoflavone excretion - Comparison between probiotic and prebiotic cohorts. Pro: n = 16, Pre: n = 14

5.2.19 Daidzein: genistein ratios and correlation in plasma and urine

To compare the levels of daidzein and genistein in plasma and urine relative to each other and their amounts in the dietary soy consumed, the ratios of daidzein: genistein (D/G) were determined. The mean plasma level of genistein was more than double that of daidzein ($D/G < 0.5$) in all samples after each 5-week dietary period (Table 5.7). There were no significant differences in the plasma D/G ratio between the control and active treatments for either cohort (probiotic: $F_{1,13} = 0.109$, $p = 0.747$, prebiotic: $F_{1,11} = 1.374$, $p = 0.266$, one-way ANOVA/RM and between groups analysis). After 5 weeks of both S + YC and S + YP, this ratio was significantly higher at 8 h compared with 24 h and after SC was significantly higher at 8 h compared with both 0 h and 24 h, while there were no significant differences between samples after S + RS (Table 5.7). For all treatments excepting the prebiotic (S + RS), the mean plasma D/G ratio was highest at 8 h and lowest at 24 h, however, after S + RS, this was not decreased 24 h post-meal. There was significant correlation between plasma daidzein and genistein at 8 h post-meal after all 5-week dietary periods, at 0 h after S + YC, S + YP and SC, and after 24 h only after S + YC and S + RS (Table 5.7).

In contrast to the plasma concentration of daidzein being less than half that of genistein, the mean urinary excretion of daidzein exceeded that of genistein in all samples. After each 5-week dietary period, there was a trend for a lower urinary D/G ratio during 0 – 24 h than 24 – 48 h, though this was not significant (Table 5.7). There were no differences between S + YC and S + YP or between SC and S + RS, ($F_{1,11} = 0.850$, $p = 0.376$ and $F_{1,10} = 6.634$, $p = 0.532$ respectively, two-way ANOVA/RM). The greater mean urinary D/G ratio between 24 and 48 h post-meal implies that more genistein had been excreted in the first 24 h post-meal. Although the plasma D/G ratio was lowest 24 h post-meal, implying that daidzein was cleared from the plasma faster than genistein, it appears genistein is excreted in urine more quickly. Daidzein and genistein were significantly correlated in urine samples at all times, except for after S + RS (Table 5.7).

Table 5.7 Ratio of daidzein: genistein in plasma and urine after each 5-week dietary period and correlations. Mean \pm SEM (range).

		Plasma *			ANOVA [†]			Urine [‡]		
		0 h	8 h	24 h	F	df	p	0 – 24 h	24 – 48 h	p
Ratio D/G	S + YC	0.35 \pm 0.08 ^{ab} (0.06 – 1.05)	0.44 \pm 0.05 ^a (0.20 – 0.83)	0.26 \pm 0.05 ^b (0.05 – 0.75)	8.077	2,12	0.006	1.94 \pm 0.17 (0.77 – 3.26)	2.50 \pm 0.54 (0.38 – 8.42)	0.226
	S + YP	0.38 \pm 0.08 ^{ab} (0.04 – 1.25)	0.42 \pm 0.05 ^a (0.23 – 1.02)	0.31 \pm 0.06 ^b (0 – 1.06)	9.242	2,14	0.003	2.20 \pm 0.22 (1.10 – 4.91)	2.41 \pm 0.32 (0.65 – 5.03)	0.158
	SC	0.20 \pm 0.04 ^a (0 – 0.46)	0.41 \pm 0.03 ^b (0.24 – 0.68)	0.24 \pm 0.04 ^a (0.07 – 0.61)	14.123	2,11	0.001	2.38 \pm 0.44 (1.00 – 7.87)	2.50 \pm 0.31 (0.95 – 5.05)	0.728
	S + RS	0.39 \pm 0.10 (0.07 – 1.50)	0.39 \pm 0.04 (0.23 – 0.87)	0.37 \pm 0.11 (0.05 – 1.64)	0.199	2,10	0.823	2.05 \pm 0.46 (0.76 – 8.24)	3.59 \pm 1.20 (0.77 – 18.89)	0.253
Correlation	S + YC	R = 0.677 p = 0.004	R = 0.611 p = 0.012	R = 0.557 p = 0.025				R = 0.568 p = 0.022	R = 0.931 p <0.0001	
	S + YP	R = 0.798 p <0.0001	R = 0.727 p = 0.001	R = 0.196 p = 0.468				R = 0.795 p <0.0001	R = 0.716 p = 0.002	
	SC	R = 0.810 p <0.0001	R = 0.696 p <0.0001	R = 0.450 p = 0.123				R = 0.876 p <0.0001	R = 0.760 p = 0.002	
	S + RS	R = 0.353 p = 0.251	R = 0.749 p = 0.002	R = 0.711 p = 0.006				R = 0.134 p = 0.635	R = 0.271 p = 0.349	

*Values in a row with different superscript letters are significantly different. $p < 0.01$, Bonferroni post-hoc analysis.

[†]One-way ANOVA with repeated measures for effect of sample time on plasma D/G ratios.

[‡]Student's paired t-test for effect of sample on urinary D/G ratios.

5.2.20 Daidzein metabolites

The occurrence of the daidzein metabolites, equol and ODMA, in plasma and urine was highly variable between subjects. Twelve subjects (4 females and 8 males) had equol in their plasma and/or urine prior to, or after, two or more of the 4 test soy meals. Of these subjects, 4 males consistently had equol in both their plasma and urine in all samples; 1 male and 1 female consistently had plasma equol, and the other 6 subjects had equol in both plasma and urine, though not as consistently. Equol was not detected in the plasma or urine of 9 subjects (4 female and 5 male) at any time during the study. Of the remaining 10 subjects (4 female and 6 male), 7 had equol in only one plasma sample, 2 had equol in urine at only one time and 1 had a low plasma equol level in one sample and low urinary equol in a different sample. This distinct separation between subjects in terms of the presence of equol in plasma and urine allowed natural classification of subjects into 3 subgroups based on equol occurrence. The 12 subjects who consistently produced equol were classified as equol-positive (eql(+)), the 10 subjects who sporadically produced equol were classified as equol-occasional (eql(o)) and the 9 subjects who never produced equol were classified as equol-negative (eql(-)). Because these subgroups are approximate tertiles of this population, this term will be used to refer to these subgroups in the rest of this chapter. There were no significant differences between these tertiles in baseline characteristics of body mass, BMI or age, nor in week 0 plasma levels or urinary concentrations of daidzein or genistein (Table 5.8).

Table 5.8 Week 0 subject characteristics and plasma and urinary daidzein and genistein for equol-tertiles. f = female, m = male.

			Eql(-)	Eql(o)	Eql(+)	F	p [†]
f / m			4 / 5	4 / 6	4 / 8		
Age			60.1 ± 2.4	57.6 ± 1.2	58.3 ± 2.9	1.270	0.297
Weight			77.1 ± 5.2	85.4 ± 3.2	81.8 ± 2.3	0.572	0.571
BMI			27.5 ± 1.0	28.7 ± 0.87	28.8 ± 0.95	0.249	0.781
Plasma (ng/mL)	daidzein	0 h	0.93 ± 0.93	3.3 ± 2.3	3.2 ± 3.2	0.272	0.764
		8 h	145 ± 17.6	178 ± 27.9	198 ± 39.5	0.686	0.512
		24 h	40.7 ± 16.9	30.8 ± 5.5	57.7 ± 16.7	1.094	0.349
	genistein	0 h	2.7 ± 2.7	2.9 ± 2.9	0	0.543	0.587
		8 h	265 ± 41.2	464 ± 72.6	442 ± 74.3	2.432	0.106
		24 h	86.3 ± 14.4	122 ± 15.9	148 ± 26.2	2.048	0.149
	equol	0 h	14.4 ± 9.7	0	0	2.004	0.154
		8 h	44.6 ± 17.6	10.0 ± 6.7	0	4.573	0.019
		24 h	20.1 ± 11.2	0	0	6.812	0.004
Urinary (mg)	daidzein	0 – 24 h	11.8 ± 1.5	11.4 ± 1.3	11.8 ± 1.4	0.031	0.969
		24 – 48 h	5.4 ± 2.6	3.1 ± 0.58	2.9 ± 0.53	1.062	0.360
	genistein	0 – 24 h	4.9 ± 0.60	6.8 ± 1.2	6.1 ± 0.91	0.893	0.421
		24 – 48 h	1.5 ± 0.98	1.4 ± 0.31	0.95 ± 0.16	0.343	0.713
	equol	0 – 24 h	1.10 ± 0.7	0	0	2.049	0.148
		24 – 48 h	0.42 ± 0.32	0.02 ± 0.02	0	1.272	0.296

[†]One-way ANOVA.

Only 2 subjects had plasma equol at week 0 and only one of these (along with 2 others) had plasma equol at week 9; correspondingly the lowest mean equol concentrations occurred at 0 h in weeks 0 and 9. The number of subjects with plasma equol increased after each 5-week dietary period of daily soy intake, although there was large variation in levels (Table 5.9). The sample times at which there were the highest numbers of subjects with plasma equol were at 24 h in week 5 and at 0 h in week 14. Equol was less prevalent in urine and there were no effects of 5 weeks daily soy intake on the number of subjects who excreted equol (Table 5.9).

Table 5.9 Number of subject with, and range of, plasma and urinary equol levels.

Week	Plasma (ng/mL)						Urine (mg)	
	0 h		8 h		24 h		0 – 24 h	24 – 48 h
	n	range	n	range	n	range	n	range
0	2	82.8 – 89.5	8	29.9 – 179	6	16.3 – 101	5	0.02 – 9.5
5	7	29.7 – 202	6	28.8 – 138	10	11.1 – 106	4	0.74 – 4.4
9	3	20.9 – 32.3	6	35.2 – 130	8	13.7 – 178	4	0.29 – 2.2
14	10	14.5 – 122	9	20.5 – 153	8	26.7 – 138	6	0.39 – 6.4

When only the equol levels of equol-positive subjects were examined (Figure 5.14), differences between the 4 test soy meals and an interaction between week and time approached significance ($F_{3,9} = 3.362$, $p = 0.069$ and $F_{6,6} = 3.761$, $p = 0.066$, respectively, two-way ANOVA/RM), due to an overall significant difference between weeks 9 and 14 ($p = 0.049$, Bonferroni post-hoc analysis). The variation in the 0 h sample was significant across the 4 weeks ($F_{3,9} = 3.737$, $p = 0.054$, one-way ANOVA/RM) with a significant difference between weeks 9 and 14 ($p = 0.033$).

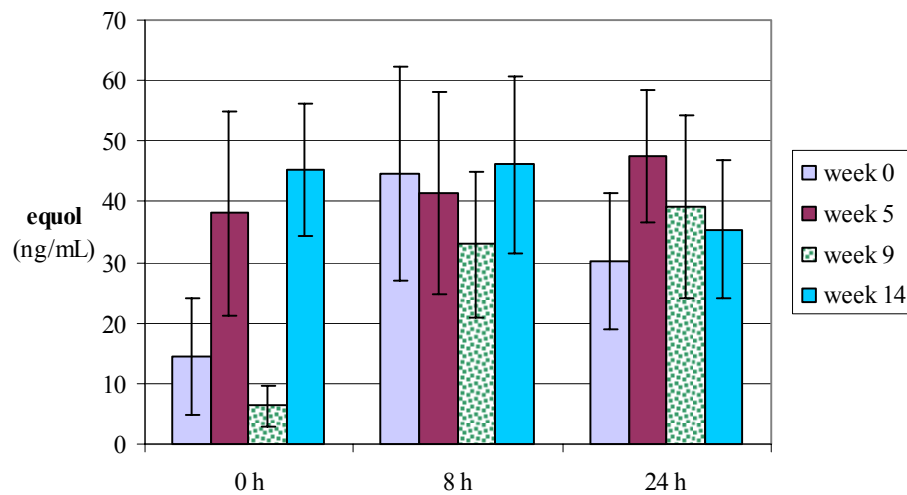


Figure 5.14 Mean plasma equol for equol-positive subjects ($n = 12$).

When separated according to probiotic or prebiotic cohort, there were 4 equol-positive males in the probiotic cohort and 8 equol-positive subjects (4 males and 4 females) in the prebiotic cohort. In the probiotic cohort, a maximum of 3 subjects had plasma equol at any one time, thus the power for statistical analysis was low. There was a mean increase in 0 h and 8 h equol with 5 weeks S + YC and a mean increase in equol concentration in all plasma samples with 5 weeks S + YP (Table 5.10). However, there were no statistical effects of either dietary period (S + YC: $F_{1,3} = 4.611$, $p = 0.121$ and S + YP: $F_{1,3} = 2.684$, $p = 0.200$, two-way ANOVA/RM) and no difference between treatments ($F_{1,3} = 1.106$, $p = 0.370$, three-way ANOVA/RM).

For equol-positive subjects in the prebiotic cohort, there were no significant effects with either treatment (SC: $F_{1,7} = 0.410$, $p = 0.543$ and S + RS: $F_{1,7} = 4.106$, $p = 0.082$, two-way ANOVA/RM). During prebiotic treatment, the plasma equol level was increased in more subjects compared with control treatment. There was an overall mean increase in 0 h and 8 h plasma equol but no change in 24 h levels with S + RS, while soy control tended to increase 0 h and 24 h levels (Table 5.10). However, there was no significant difference between treatments ($F_{1,7} = 0.405$, $p = 0.545$, three-way ANOVA/RM).

Urinary excretion of equol was variable and only up to 4 subjects from probiotic and prebiotic cohorts excreted equol at any given time. Five weeks of S + YC tended to decrease equol excretion between 0 – 24 h, but increased it between 24 – 48 h, while 5 weeks S + YP tended to increase urinary equol excretion in both samples. In the prebiotic cohort, both dietary periods (SC and S + RS) tended to increase both 0 – 24 h and 24 – 48 h urinary equol excretion (Table 5.10).

Table 5.10 Plasma and urinary equol levels for equol-positive subjects of probiotic and prebiotic cohorts. Mean \pm SEM.

Cohort	Treatment	Wk	Plasma equol (ng/mL)			Urinary equol (mg)	
			0 h	8 h	24 h	0 – 24 h	24 – 48 h
Probiotic	S + YC	0	0	37.5 \pm 25.5	46.1 \pm 26.9	2.4 \pm 2.4	1.1 \pm 1.0
		5	49.7 \pm 17.7	53.8 \pm 32.6	31.7 \pm 18.8	1.2 \pm 1.1	1.3 \pm 1.0
	S + YP	0	26.4 \pm 19.6	49.3 \pm 30.4	39.7 \pm 13.5	1.1 \pm 0.47	0.51 \pm 0.40
		5	69.9 \pm 45.0	60.1 \pm 34.8	64.0 \pm 31.2	1.9 \pm 1.5	2.0 \pm 1.2
Prebiotic	SC	0	11.2 \pm 11.2	43.6 \pm 20.8	31.1 \pm 13.3	0.83 \pm 0.71	0.28 \pm 0.15
		5	37.2 \pm 10.5	30.4 \pm 16.8	46.9 \pm 11.8	1.2 \pm 0.68	0.35 \pm 0.14
	S + RS	0	6.7 \pm 4.5	29.5 \pm 17.0	29.8 \pm 21.5	0.44 \pm 0.43	0.80 \pm 0.65
		5	28.2 \pm 16.2	44.0 \pm 18.6	29.6 \pm 10.6	0.85 \pm 0.85	0.95 \pm 0.54

When subjects were separated into the equol tertiles, there was a trend for mean plasma daidzein and genistein to be lowest in eql(-) subjects and highest in eql(+) subjects (Figure 5.15), however there were no significant differences between the tertiles ($F = 1.113$, $p = 0.343$ for daidzein and $F = 2.228$, $p = 0.127$ for genistein, two-way ANOVA/RM and between groups analysis). There were no significant differences or trends for urinary excretion of daidzein and genistein between the tertiles ($F = 0.157$, $p = 0.856$ and $F = 0.315$, $p = 0.732$ respectively, two-way ANOVA/RM and between groups analysis, Figure 5.16).

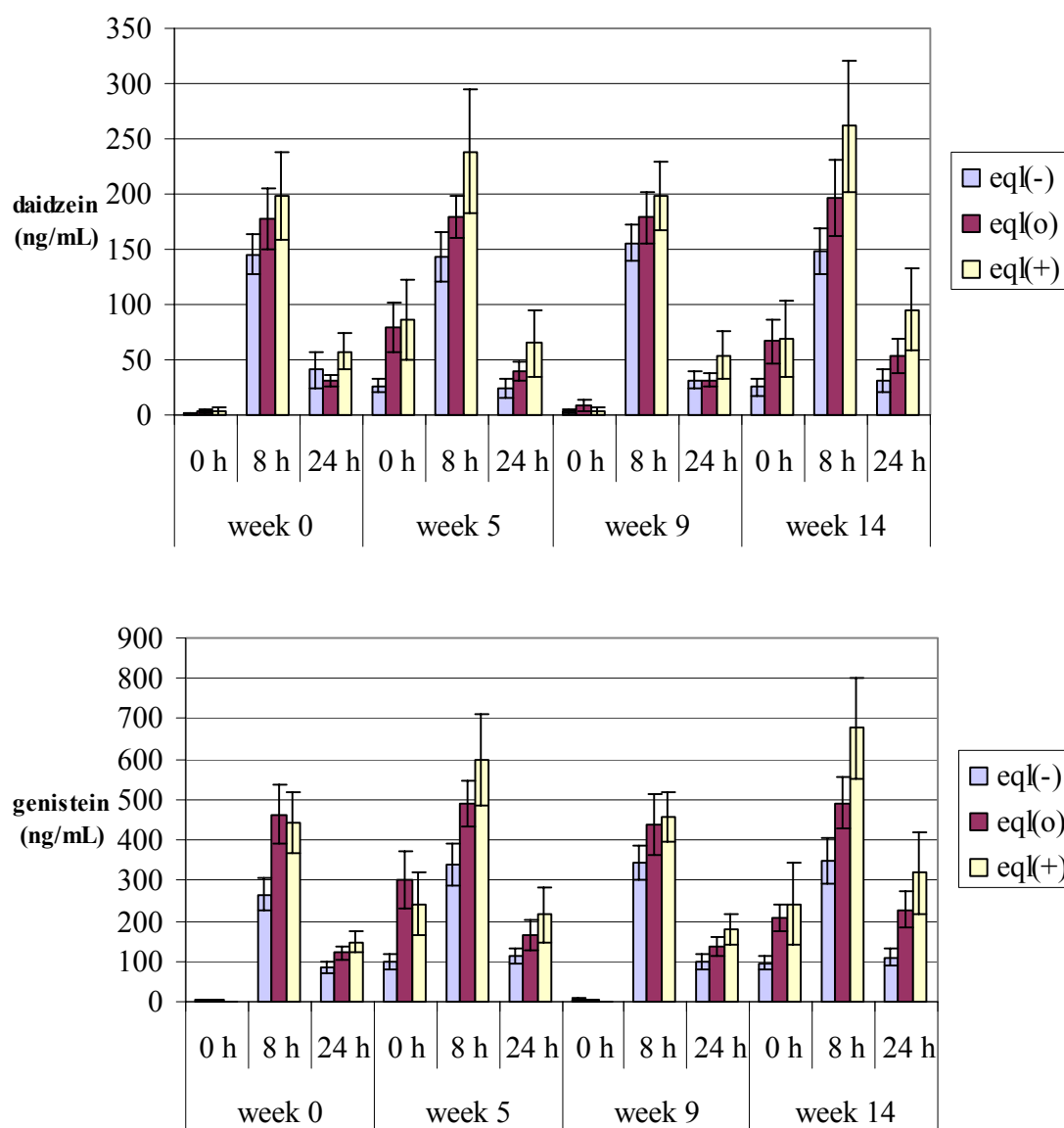


Figure 5.15 Mean plasma daidzein and genistein for subjects in equol tertiles.

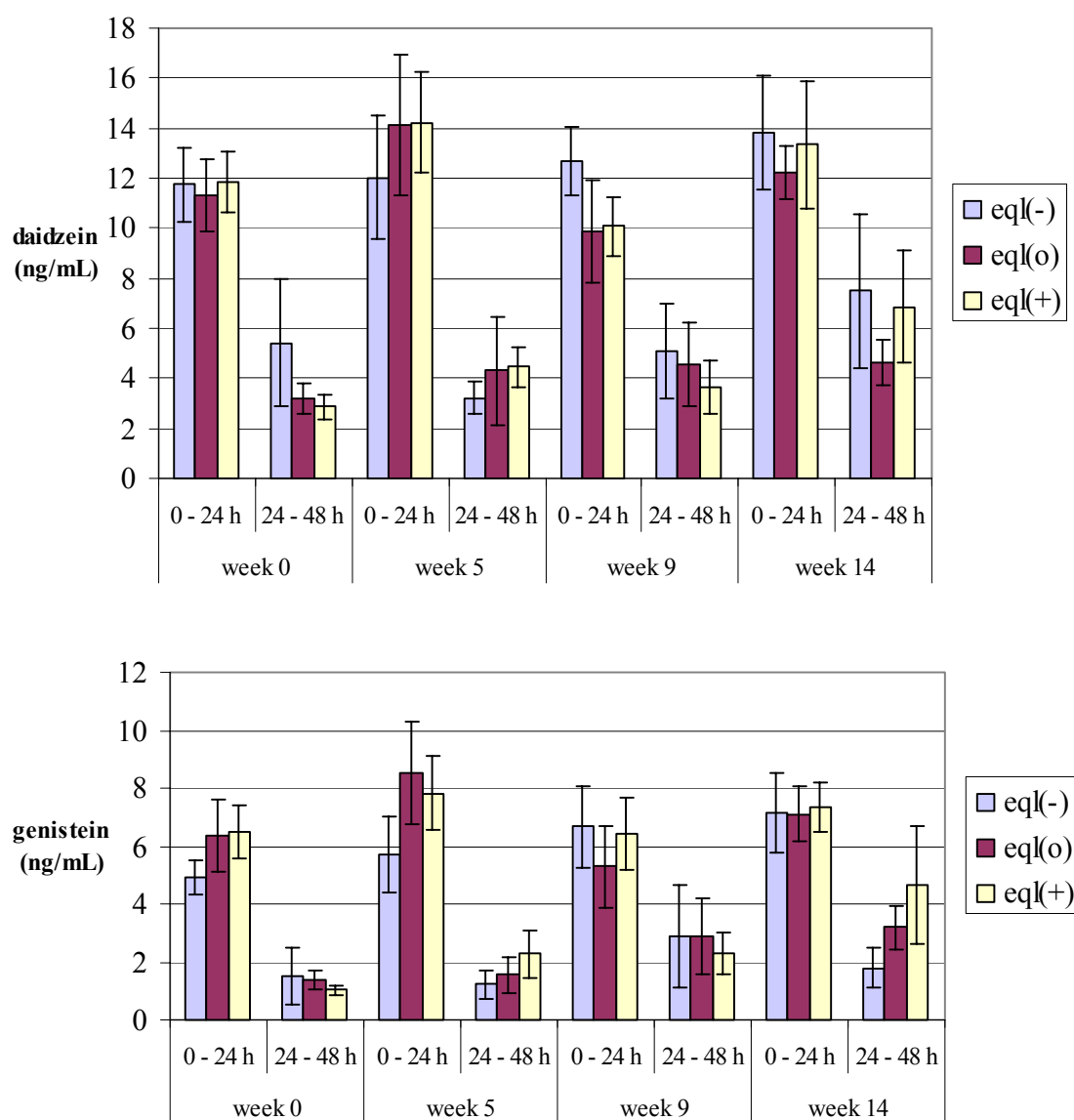


Figure 5.16 Mean urinary daidzein and genistein excretion for subjects in equol tertiles.

To determine whether the equol status of a subject determined how they responded to any of the dietary treatments, plasma daidzein and genistein levels at 0 h in week 5 of each treatment were examined. This was chosen as a marker because it was the only measure to have increased significantly with each 5-week dietary period. The 0 h plasma levels of daidzein and genistein after the 4 different dietary treatments (S + YC, S + YP, SC and S + RS) were very variable between equol tertiles (Table 5.11). The only significant influence of equol classification was for 0 h genistein after SC, for which plasma levels were significantly greater in eql(o) compared with both eql(-) and eql(+) subjects ($p = 0.023$ and $p = 0.028$ respectively, Bonferroni post-hoc analysis). Apart from this sole significant difference, there was a trend for mean 0 h plasma daidzein and genistein to have been greatest in eql(o) subjects after each 5 week dietary period except for daidzein after S + RS.

Table 5.11 Mean 0 h plasma daidzein and genistein levels after 5 weeks of each treatment for equol tertiles.

		Eql(-)		Eql(o)		Eql(+)		F	p [†]
		n	ng/mL	n	ng/mL	n	ng/mL		
daidzein	S + YC	4	21.3 ± 8.8	6	102 ± 45.1	6	15.1 ± 2.9	2.324	0.137
	S + YP	4	36.5 ± 8.2	6	105 ± 33.5	6	69.7 ± 55.8	2.236	0.146
	SC	8	23.3 ± 8.6	4	92.6 ± 36.8	3	34.9 ± 19.2	2.591	0.120
	S + RS	8	41.1 ± 12.8	4	33.2 ± 7.5	3	55.7 ± 22.4	0.347	0.714
genistein	S + YC	4	87.3 ± 28.1	6	270 ± 104	6	141 ± 34.6	1.089	0.365
	S + YP	4	105 ± 16.5	6	298 ± 74.7	6	248 ± 146	1.944	0.183
	SC	8	121 ± 27.2	4	435 ± 161	3	142 ± 25.8	6.265	0.015
	S + RS	8	93.7 ± 17.1	4	235 ± 53.3	3	160 ± 51.1	2.073	0.172

[†]One way ANOVA.

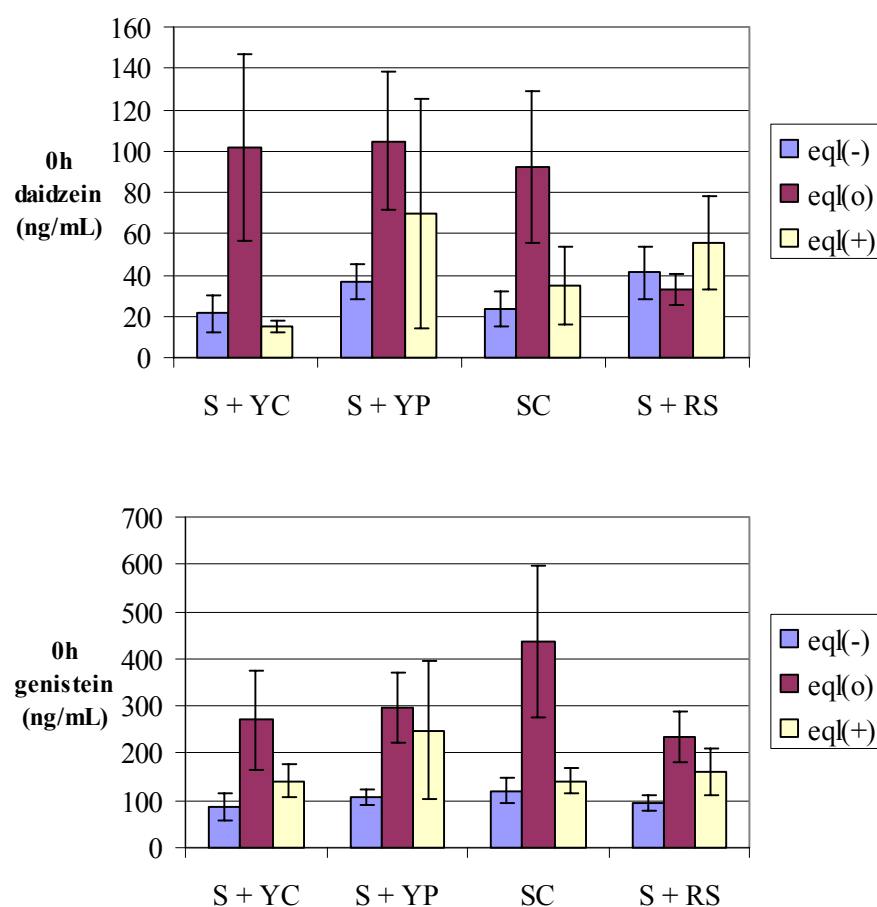


Figure 5.17 Mean 0 h plasma daidzein and genistein levels after 5 weeks of each treatment for equol tertiles.

ODMA was present in the plasma of only 2 subjects during the study; in one subject ODMA occurred in a single plasma sample while the other subject had plasma ODMA throughout the study. The latter subject (PB06) was eql(-) and never excreted ODMA in their urine. Furthermore, their plasma daidzein concentration was mostly less than the corresponding mean plasma daidzein concentration and was exceeded by the plasma concentration of ODMA (Table 5.8). The former subject (PB40) only had plasma ODMA at 0 h in week 5 (375 ng/mL after 5 weeks S + YC), which was also the only sample of theirs (plasma or urine) that contained equol (73.1 ng/mL). Their plasma

daidzein concentration was also the highest at this time of all subjects in the probiotic cohort (261 ng/mL compared with the mean level of 45.0 ng/mL). Six subjects had detectable ODMA in their urine, in up to 5 samples and at levels between 0.23 and 1.4 mg. For one subject who only excreted ODMA in one sample, this was the same and only urine sample of theirs that also contained equol; another subject who excreted ODMA at 2 time points was eql(+); the other 4 subjects were eql(-).

Table 5.12 Comparison between plasma levels of ODMA and daidzein for subject PB 06 and daidzein levels of the probiotic cohort (ng/mL).

	Week 0			Week 5		
	0 h	8 h	24 h	0 h	8 h	24 h
S + YC						
mean daidzein	1.9 ± 1.5	169 ± 19.3	38.3 ± 8.5	45.0 ± 16.8	193 ± 22.7	41.2 ± 12.0
PB 06 daidzein	0	145	19.7	20.1	100	8.3
PB 06 ODMA	0	383	421	457	182	335
S + YP						
mean daidzein	3.0 ± 2.2	176 ± 20.4	30.3 ± 5.7	66.2 ± 17.8	154 ± 20.8	34.5 ± 5.9
PB 06 daidzein	0	85.2	35.6	27.6	112	18.1
PB 06 ODMA	0	0	292	390	0	73.9

5.2.21 Gender differences in plasma and urinary isoflavones

Females had significantly higher concentrations of plasma daidzein than males at week 0, but there was no gender difference in plasma genistein (one-way ANOVA/RM and between groups analysis, Table 5.13). There was a significant interaction between sample and gender for daidzein ($F_{2,26} = 4.592$, $p = 0.020$), due to females having higher plasma daidzein levels at 8 h and 24 h, but not at 0 h compared with males (Table 5.13). There was an interaction between sample and gender that approached significance for plasma genistein ($F_{2,26} = 3.148$, $p = 0.060$). This was due to females having near significantly higher plasma genistein at 8 h ($p = 0.036$), but no significant differences at either 0 h or 24 h ($p = 0.286$ and $p = 0.914$ respectively, Student's paired t-tests with a Bonferroni-adjusted level of significance of $p = 0.017$). In contrast to the plasma, there

were no statistical differences between males and females in urinary excretion of either daidzein or genistein at week 0 ($F = 1.555$, $p = 0.223$ and $F < 0.0001$, $p = 0.992$ respectively, one-way ANOVA/RM and between groups analysis). There was a trend for urinary excretion of daidzein to be greater in females, but not for genistein (Table 5.13).

Table 5.13 Gender differences in plasma and urinary daidzein and genistein for samples at week 0. f = females, n = 11; m = males, n = 19.

			females	males*	F	p [†]
plasma (ng/mL)	daidzein	0 h	1.2 ± 1.2	1.7 ± 1.3	8.386	0.007
		8 h	213 ± 27.8 ^a	137 ± 13.6 ^b		
		24 h	57.2 ± 12.5 ^a	26.5 ± 4.9 ^b		
	genistein	0 h	0	2.9 ± 2.0	2.859	0.102
		8 h	501 ± 75.0	325 ± 42.8		
		24 h	117 ± 14.5	114 ± 15.4		
urine (mg)	daidzein	0 – 24 h	12.9 ± 1.3	10.9 ± 0.93	1.555	0.223
		24 – 48 h	4.6 ± 1.7	3.0 ± 0.48		
	genistein	0 – 24 h	6.0 ± 0.91	6.0 ± 0.72	0.000	0.992
		24 – 48 h	1.4 ± 0.65	1.2 ± 0.21		

[†]One-way ANOVA/RM and between groups analysis.

*Values in same row with different superscripts are significantly different (post-hoc t-tests with Bonferroni correction of $p < 0.017$).

To examine whether the gender difference in weight (Table 5.2) may have accounted for gender differences in plasma isoflavones, week 0 body mass was correlated with plasma daidzein and genistein values for all subjects and for each gender separately (Table 5.14). For all subjects, there was a significant inverse correlation between 24 h daidzein and weight, independent of gender, and there was a trend approaching significance for a correlation between body mass and daidzein in females. Body mass was also inversely correlated with age ($R = -0.471$, $p = 0.009$, $n = 30$). However, the same correlation was not significant for either gender alone (females: $R = -0.427$, $p = 0.167$ and males: $R = -0.395$, $p = 0.095$). To determine if age was a determinant of plasma isoflavone levels, this measure was also used in correlation analysis and there was high positive correlation between age and 24 h daidzein for all subjects and for female subjects (Table 5.14). It should be noted that the female subject whose plasma isoflavone data were originally

removed from analyses due to the unusually high levels was also the eldest subject, further exemplifying this trend for increased daidzein with age, though this subject's data were still not included in these analyses.

Table 5.14 Correlations between weight and plasma isoflavones and between age and plasma isoflavones. R = Pearson's correlation coefficient.

			daidzein		genistein	
		n	8 h	24 h	8 h	24 h
weight	all subjects	30	R = -0.249 p = 0.185	R = -0.447 p = 0.015	R = -0.184 p = 0.329	R = -0.275 p = 0.149
	females	11	R = 0.189 p = 0.578	R = -0.547 <i>p = 0.081</i>	R = -0.096 p = 0.778	R = -0.184 p = 0.588
	males	19	R = -0.268 p = 0.267	R = -0.003 p = 0.990	R = 0.144 p = 0.557	R = -0.069 p = 0.784
age	all subjects	30	R = 0.251 p = 0.180	R = 0.517 p = 0.004	R = 0.192 p = 0.310	R = 0.204 p = 0.289
	females	11	R = 0.512 p = 0.532	R = 0.683 p = 0.021	R = 0.302 p = 0.367	R = 0.195 p = 0.566
	males	19	R = 0.153 p = 0.531	R = 0.343 p = 0.164	R = -0.035 p = 0.887	R = 0.103 p = 0.684

To determine whether the four dietary treatments affected plasma daidzein or genistein differently between genders, these were compared within each cohort (statistics are reported as one-way ANOVA/RM and between groups analysis; graphs are included as Appendix 5.7). Overall, mean plasma levels of daidzein were higher in females than males, either significantly or as a trend. For the probiotic cohort, there was a trend for greater plasma daidzein in females during S + YC and S + YP ($F = 3.205$, $p = 0.095$ and $F = 2.012$, $p = 0.178$, respectively), but no significant gender difference (or trend) for plasma genistein ($F = 0.984$, $p = 0.338$ and $F = 0.016$, $p = 0.901$, respectively). For combined prebiotic groups, plasma daidzein was significantly higher in females during SC, and showed a trend towards being higher during S + RS ($F = 6.353$, $p = 0.028$ and $F = 1.985$, $p = 0.187$ respectively). Plasma genistein was not significantly different for either treatment (S – RS: $F = 2.890$, $p = 0.117$; S + RS: $F = 0.574$, $p = 0.464$).

Although there were no significant differences between males and females in urinary isoflavone excretion through the study, the genders showed different trends according to the treatment. There were no gender effects on urinary daidzein or genistein excretion for the probiotic cohort with either S + YC ($F = 1.624$, $p = 0.223$ and $F = 0.008$, $p = 0.932$ respectively) or S + YP ($F = 0.269$, $p = 0.612$ and $F = 0.002$, $p = 0.962$ respectively). There was a trend however for 0 – 24 h urinary excretion of daidzein and genistein to have increased but that between 24 and 48 h to have decreased in females with S + YC. The urinary excretion of both isoflavones increased more in males compared with females with S + YP.

In the prebiotic cohort, there were no significant overall gender differences in urinary daidzein or genistein with SC ($F = 2.167$, $p = 0.167$ and $F = 0.463$, $p = 0.509$, respectively), but there was an interaction between week and gender as both isoflavones increased in females over the 5 week period but were not altered in males ($F_{1,12} = 4.946$, $p = 0.046$ for daidzein and $F_{1,12} = 4.735$, $p = 0.050$ for genistein, Figure 5.21). During the prebiotic treatment, there were no significant gender differences ($F = 0.025$, $p = 0.878$ and $F = 0.269$, $p = 0.613$ for daidzein and genistein, respectively), but a trend for isoflavones to have increased during the 5 weeks in males and decreased in females.

5.2.22 Lipids: baseline levels and changes through study

There were four subjects in Pro-1 whose total cholesterol levels were especially high at each of the four sample times resulting in a higher mean cholesterol level for this group (Table 5.15). One of these subjects also had exceptionally high triglyceride levels throughout the study (> 8.2 mmol/L) and was excluded from all lipid analyses. Within each group, distribution was normal for each of the four lipid measures and there were no statistical differences between any of the groups for week 0 levels (one-way ANOVA between groups, Table 5.15). Based on the National Heart Foundation of Australia guidelines, subjects were classified as hyperlipidemic at baseline (TCh > 6.0 mmol/L, LDL > 4.0 mmol/L), but had healthy HDL levels (> 1.0 mmol/L) and most were in the recommended range for triglyceride levels (< 2.0 mmol/L).

Table 5.15 Lipid levels (mmol/L) at baseline for each group and the whole study.
Mean \pm SEM.

	n	TCh	HDL	LDL	TG
Pro-1	9	7.07 \pm 0.48	1.29 \pm 0.13	5.00 \pm 0.49	1.71 \pm 0.18
Pro-2	6	6.31 \pm 0.22	1.12 \pm 0.15	4.09 \pm 0.28	2.41 \pm 0.48
Pre-1	9	6.72 \pm 0.20	1.30 \pm 0.09	4.50 \pm 0.26	2.02 \pm 0.26
Pre-2	7	6.29 \pm 0.24	1.27 \pm 0.11	4.13 \pm 0.29	1.96 \pm 0.22
Between groups		F = 1.233 p = 0.317	F = 0.413 p = 0.745	F = 1.376 p = 0.271	F = 1.007 p = 0.405
Total	31	6.65 \pm 0.17	1.26 \pm 0.06	4.48 \pm 0.18	1.99 \pm 0.14

For the four study groups, there were few significant changes in lipids (Table 5.17). There were no significant differences between the two probiotic groups for any lipid measures (Table 5.16), thus groups were combined for further analysis. However, there were significant differences between groups of the prebiotic cohort for total and LDL cholesterol levels during SC (Table 5.16), as Pre-2 had lower levels of both in week 0 and week 5 of SC. Pre-2 followed the SC dietary period after the S + RS dietary period which had already significantly reduced lipid levels and these had remained lowered during the washout (Table 5.17). Therefore, the prebiotic groups were initially analysed separately, however as both groups showed similar trends, they were also combined for further analysis.

Table 5.16 Statistics for between groups analysis for lipid levels. One-way ANOVA with repeated measures and between-groups analysis.

Between groups	Treatment	TCh	HDL	LDL	TG
Pro-1 & Pro-2	S + YC	F = 2.056 p = 0.175	F = 1.012 p = 0.333	F = 2.453 p = 0.141	F = 1.997 p = 0.181
	S + YP	F = 0.621 p = 0.445	F = 1.125 p = 0.308	F = 1.193 p = 0.295	F = 3.112 p = 0.101
Pre-1 & Pre-2	SC	F = 7.269 p = 0.018	F = 0.080 p = 0.782	F = 4.846 p = 0.046	F = 0.013 p = 0.910
	S + RS	F = 2.065 p = 0.173	F = 0.001 p = 0.919	F = 2.099 p = 0.169	F = 0.347 p = 0.565

Table 5.17 Lipid Changes During Study for each Study Group.

		Lipid Level * (mmol/L)				ANOVA [†]			Weeks 0 - 5		Weeks 9 - 14	
		wk 0	wk 5	wk 9	wk 14	F	df	p	Δ	% Δ	Δ	% Δ
Pro-1	TCh	7.07 ± 0.48	6.98 ± 0.45	6.95 ± 0.48	6.52 ± 0.51	5.199	3,6	0.042	-0.09 ± 0.16	-0.9 ± 2.2	-0.43 ± 0.21	-6.3 ± 2.9
	HDL	1.29 ± 0.13	1.32 ± 0.12	1.32 ± 0.10	1.20 ± 0.09	1.186	3,6	0.391	0.03 ± 0.03	3.6 ± 3.4	-0.12 ± 0.08	-7.7 ± 5.2
	LDL	5.00 ± 0.49	4.90 ± 0.44	4.81 ± 0.46	4.63 ± 0.50	2.497	3,6	0.157	-0.10 ± 0.10	-1.5 ± 2.2	-0.18 ± 0.15	-4.0 ± 3.4
	TG	1.71 ± 0.18	1.91 ± 0.25	1.81 ± 0.21	1.65 ± 0.17	0.533	3,6	0.676	0.20 ± 0.19	13.6 ± 11.3	-0.16 ± 0.18	-4.3 ± 8.3
Pro-2	TCh	6.31 ± 0.22	6.17 ± 0.29	6.25 ± 0.25	6.09 ± 0.23	1.134	3,3	0.460	-0.14 ± 0.14	-2.4 ± 2.3	-0.16 ± 0.18	-2.4 ± 3.0
	HDL	1.12 ± 0.15	1.06 ± 0.16	1.15 ± 0.15	1.07 ± 0.15	0.852	3,3	0.551	-0.06 ± 0.06	-4.8 ± 6.4	-0.07 ± 0.04	-6.1 ± 4.2
	LDL	4.09 ± 0.28	3.95 ± 0.35	4.09 ± 0.29	3.89 ± 0.28	0.577	3,3	0.669	-0.14 ± 0.14	-3.8 ± 3.6	-0.20 ± 0.22	-4.1 ± 5.9
	TG	2.41 ± 0.48	2.73 ± 0.61	2.40 ± 0.76	2.63 ± 0.36	1.191	3,3	0.445	0.32 ± 0.20	11.4 ± 7.5	0.23 ± 0.48	35.3 ± 20.9
Pre-1	TCh	6.72 ± 0.20	6.51 ± 0.16	6.88 ± 0.13	6.37 ± 0.27	7.357	3,6	0.050	-0.22 ± 0.10	-3.1 ± 1.4	-0.50 ± 0.17	-7.6 ± 2.6
	HDL	1.30 ± 0.09	1.28 ± 0.09	1.30 ± 0.09	1.24 ± 0.07	0.582	3,6	0.648	-0.02 ± 0.03	-1.3 ± 2.6	-0.05 ± 0.05	-3.0 ± 3.2
	LDL	4.50 ± 0.26	4.27 ± 0.20 ^a	4.68 ± 0.17 ^b	4.30 ± 0.27	7.801	3,6	0.017	-0.24 ± 0.12	-4.5 ± 2.8	-0.38 ± 0.16	-8.6 ± 3.7
	TG	2.02 ± 0.26	2.10 ± 0.30	1.99 ± 0.22	1.84 ± 0.12	0.295	3,6	0.828	0.08 ± 0.20	6.7 ± 10.0	-0.15 ± 0.20	-0.5 ± 11.2
Pre-2	TCh	6.29 ± 0.25	6.11 ± 0.21	5.88 ± 0.20	5.98 ± 0.15	2.227	3,3	0.264	-0.18 ± 0.07	-2.7 ± 1.0	0.10 ± 0.08	1.8 ± 1.5
	HDL	1.27 ± 0.11	1.24 ± 0.09	1.26 ± 0.10	1.25 ± 0.15	0.242	3,3	0.863	-0.03 ± 0.05	-1.0 ± 4.0	-0.01 ± 0.07	-2.2 ± 5.3
	LDL	4.13 ± 0.29	3.88 ± 0.25	3.81 ± 0.22	3.63 ± 0.12	1.782	3,3	0.323	-0.25 ± 0.08	-5.7 ± 1.8	-0.17 ± 0.13	-3.6 ± 3.5
	TG	1.96 ± 0.22	2.17 ± 0.28	1.80 ± 0.17	2.41 ± 0.41	1.387	3,3	0.397	0.21 ± 0.20	11.6 ± 10.7	0.61 ± 0.36	33.1 ± 19.4

[†] One-way ANOVA with repeated measures

*Values in a row with different superscript letters are significantly different. p < 0.01, Bonferroni post-hoc analysis

5.2.23 Probiotic effects on lipids

For the probiotic cohort, there was a significant reduction in total cholesterol levels after S + YP but no other significant changes with either S + YC or S + YP (Student's paired t-tests, Table 5.18). There was a trend however, for a reduction of HDL cholesterol with S + YP and for a reduction in LDL with both treatments. There were no significant differences between the two treatments on any of the lipid measures (Table 5.18).

Table 5.18 Effects of 5 weeks soy + control yoghurt (S + YC) and soy + probiotic yoghurt (S + YP) on lipids in probiotic cohort. Mean \pm SEM, n = 15

		Lipid Level (mmol/L)		p^{\dagger}	Change in Lipid		ANOVA [‡]	
		Week 0	Week 5		(mmol/L)	(%)	F _{1,14}	p
TCh	S + YC	6.74 \pm 0.32	6.62 \pm 0.30	0.324	-0.12 \pm 0.12	-1.5 \pm 1.7	1.478	0.244
	S + YP	6.70 \pm 0.30	6.38 \pm 0.32	0.038	-0.32 \pm 0.14	-4.7 \pm 2.0		
HDL	S + YC	1.23 \pm 0.10	1.22 \pm 0.10	0.663	-0.01 \pm 0.03	-0.3 \pm 2.8	0.689	0.420
	S + YP	1.24 \pm 0.09	1.15 \pm 0.08	0.084	-0.09 \pm 0.05	-6.5 \pm 3.9		
LDL	S + YC	4.64 \pm 0.33	4.49 \pm 0.31	0.192	-0.14 \pm 0.10	-2.5 \pm 2.6	1.879	0.192
	S + YP	4.52 \pm 0.30	4.36 \pm 0.33	0.133	-0.17 \pm 0.10	-3.9 \pm 2.4		
TG	S + YC	1.99 \pm 0.32	2.20 \pm 0.22	0.334	0.21 \pm 0.21	22.3 \pm 10.7	0.050	0.827
	S + YP	2.05 \pm 0.23	2.08 \pm 0.29	0.834	0.03 \pm 0.15	2.0 \pm 6.0		

[†]Paired t-tests for comparison between week 0 and week 5 of each treatment.

[‡]Two-way ANOVA with repeated measures for comparison between treatments.

5.2.24 Prebiotic effects on lipid levels

During the control soy dietary period, Pre-1 showed a trend towards a reduction in total cholesterol (p = 0.058) but there were no other significant effects of this treatment in either prebiotic group (Table 5.19). S + RS significantly reduced total and LDL cholesterol levels in both groups (Pre-1: p = 0.017 and p = 0.049, respectively and Pre-2: p = 0.036 and p = 0.022, respectively, Student's paired t-tests). When prebiotic groups were combined, there were highly significant reductions in total and LDL cholesterol levels with S + RS and a significant reduction of LDL with SC (Table 5.19). Although

mean TG appeared to increase during the SC period but not during S + RS, variation was high and this was not significant. There were no significant differences between the treatments in their effects on any of the lipid measures (Table 5.19).

Table 5.19 Effects of 5 weeks control soy (SC) and of soy + resistant starch (S + RS) on lipids in prebiotic cohort. Mean \pm SEM, n = 15.

		Lipid Level (mmol/L)		p [†]	Change in Lipid		ANOVA [‡]	
		Week 0	Week 5		(mmol/L)	(%)	F _{1,14}	p [†]
TCh	SC	6.39 \pm 0.18	6.29 \pm 0.13	0.250	-0.09 \pm 0.08	-1.1 \pm 1.8	0.433	0.521
	S + RS	6.62 \pm 0.15	6.26 \pm 0.18	0.003	-0.36 \pm 0.10	-5.5 \pm 1.6		
HDL	SC	1.29 \pm 0.07	1.27 \pm 0.08	0.582	-0.02 \pm 0.03	-1.7 \pm 2.5	0.019	0.891
	S + RS	1.29 \pm 0.07	1.24 \pm 0.05	0.200	-0.04 \pm 0.03	-2.1 \pm 2.4		
LDL	SC	4.22 \pm 0.19	4.01 \pm 0.15	0.030	-0.21 \pm 0.09	-4.1 \pm 2.1	0.969	0.342
	S + RS	4.44 \pm 0.17	4.11 \pm 0.19	0.005	-0.32 \pm 0.10	-7.3 \pm 2.2		
TG	SC	1.93 \pm 0.17	2.22 \pm 0.24	0.153	0.29 \pm 0.19	17.2 \pm 10.0	0.270	0.611
	S + RS	1.97 \pm 0.15	1.98 \pm 0.14	0.946	0.01 \pm 0.15	4.8 \pm 7.7		

[†]Paired t-tests for comparison between week 0 and week 5 of each treatment.

[‡]Two-way ANOVA with repeated measures for comparison between treatments.

5.2.25 Comparison between probiotic and prebiotic groups for lipids

For all four groups, there was an overall reduction in both total and LDL-cholesterol over the 14 weeks (Table 5.17), suggesting some lipid-lowering effects of soy intake. The only significant lipid reductions were of total cholesterol with 5 weeks S + YP and S + RS and of LDL with 5 weeks of SC and S + RS (Figure 5.22). However, there did appear to be some influence of the order of probiotic or prebiotic intake on some of the lipid effects. Although TCh and LDL were reduced in Pre-1 during weeks 0 – 5, this was reversed during the washout, but these lipid levels in Pre-2 continued to decrease during the washout. Thus, there appears to be a longer-lasting effect of cholesterol reduction with S + RS. Further, there was a trend for a mean increase in TG with both soy control dietary periods, but there were no increases with S + YP and S + RS.

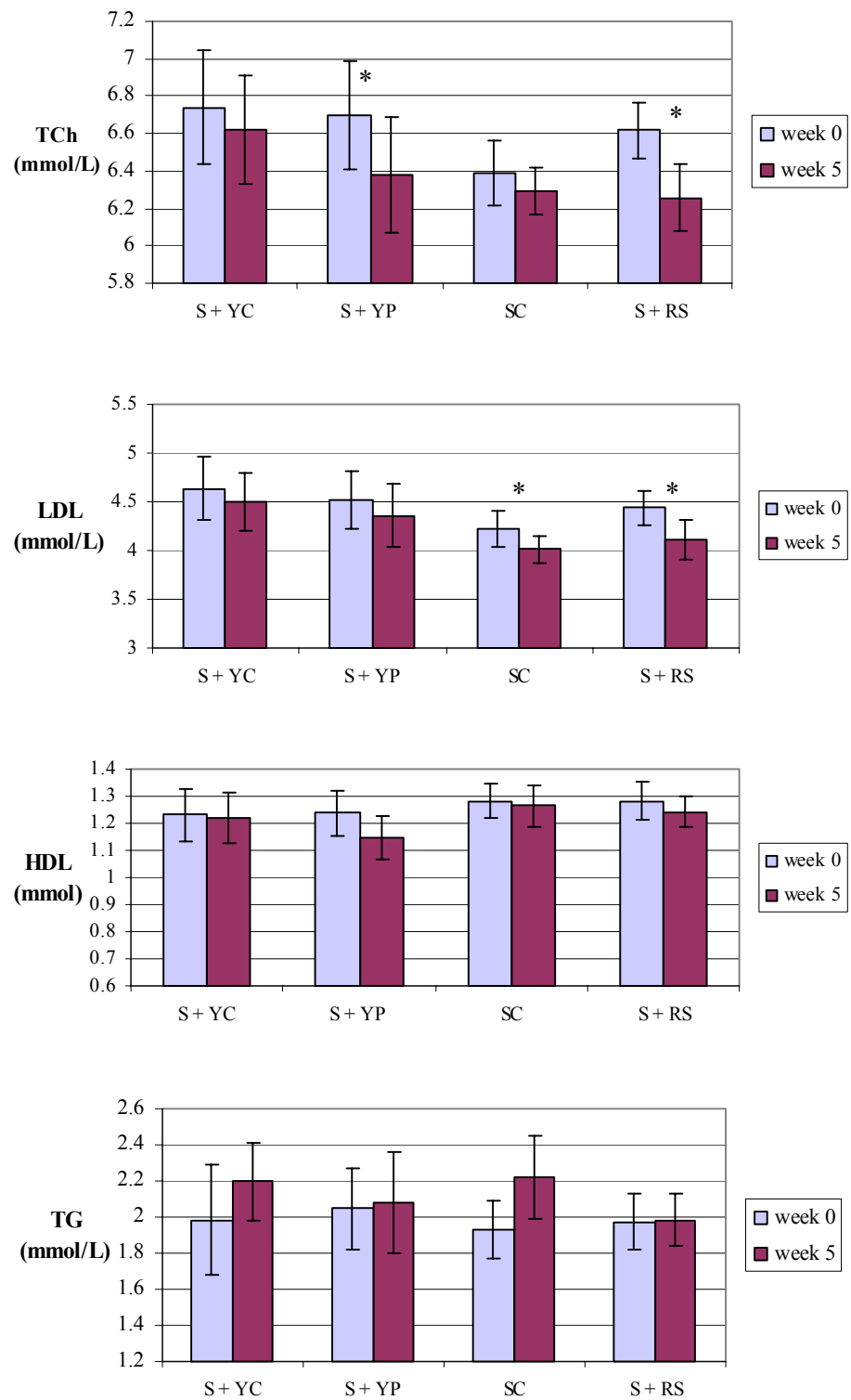


Figure 5.18 Mean lipids for each dietary period. Mean \pm SEM.

The only significant inverse correlations between baseline lipids and subsequent changes during the dietary periods were between baseline TCh and subsequent changes in TCh and LDL during SC, between baseline LDL and change in LDL with SC, between HDL and TG and subsequent changes in their respective levels with S + RS and between TG and the change in TG with S + YC (Table 5.20). Correlations between change in TCh and baseline TCh levels for SC and S + YC are presented in

Table 5.20 Correlation between baseline lipid levels and subsequent changes with dietary treatment. R = Pearson's correlation coefficient.

Correlation		S + YC		S + YP		SC		S + RS	
		R	p	R	p	R	p	R	p
TCh	Δ TCh	-0.309	0.262	-0.092	0.744	-0.751	0.001	-0.032	0.905
	Δ LDL	-0.236	0.398	0.104	0.713	-0.501	0.057	0.028	0.918
LDL	Δ LDL	-0.343	0.210	0.103	0.716	-0.687	0.005	-0.050	0.853
HDL	Δ HDL	-0.260	0.349	-0.379	0.163	0.125	0.656	-0.586	0.017
TG	Δ TG	-0.726	0.002	-0.272	0.327	-0.135	0.632	-0.550	0.027

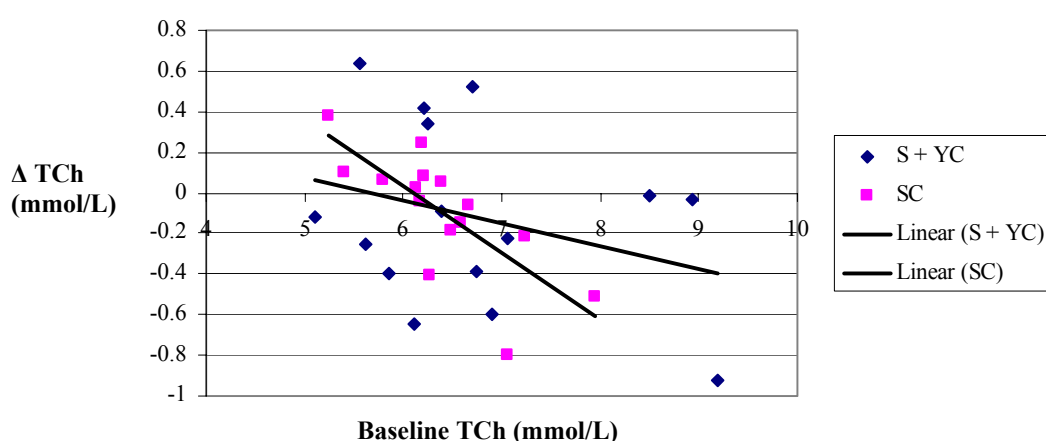


Figure 5.19 Correlation between baseline TCh and change in TCh with soy dietary period for control of probiotic cohort (S + YC) and control of prebiotic cohort (SC).

5.2.26 Relation between equol and lipids

There were no significant differences in week 0 lipids between equol tertiles and the only significant difference between groups during the study was for the effect of S + YP on LDL (Table 5.21), for which only eql(-) subjects had a mean decrease.

Table 5.21 Week 0 Lipids and lipid changes with each dietary period for equol-tertiles.

		Eql(-)	Eql(o)	Eql(+)	F	p[†]
Week 0	TCh	6.49 ± 0.34	6.68 ± 0.37	6.70 ± 0.25	0.130	0.878
	HDL	1.37 ± 0.08	1.13 ± 0.13	1.27 ± 0.10	1.154	0.330
	LDL	4.31 ± 0.30	4.57 ± 0.39	4.48 ± 0.32	0.140	0.870
	TG	1.79 ± 0.21	2.14 ± 0.27	2.08 ± 0.25	0.511	0.606
S + YC	Δ TCh	0.03 ± 0.19	0.00 ± 0.12	-0.50 ± 0.17	2.389	0.134
	Δ HDL	-0.04 ± 0.04	-0.04 ± 0.03	0.05 ± 0.07	0.949	0.414
	Δ LDL	-0.16 ± 0.18	-0.08 ± 0.15	-0.17 ± 0.24	0.061	0.941
	Δ TG	0.50 ± 0.15	0.23 ± 0.13	-0.31 ± 0.75	1.279	0.314
S + YP	Δ TCh	-0.57 ± 0.19	-0.34 ± 0.26	0.16 ± 0.12	3.152	0.079
	Δ HDL	-0.16 ± 0.10	-0.07 ± 0.08	0.00 ± 0.04	0.819	0.464
	Δ LDL	-0.48 ± 0.11 ^a	0.10 ± 0.12 ^b	0.12 ± 0.16 ^b	7.947	0.006
	Δ TG	0.16 ± 0.21	-0.24 ± 0.40	0.08 ± 0.16	0.605	0.562
SC	Δ TCh	0.22 ± 0.11	-0.26 ± 0.13	-0.13 ± 0.10	2.912	0.093
	Δ HDL	0.04 ± 0.11	0.06 ± 0.08	-0.08 ± 0.13	2.543	0.120
	Δ LDL	0.17 ± 0.26	-0.31 ± 0.09	-0.24 ± 0.13	0.832	0.435
	Δ TG	0.35 ± 0.83	-0.02 ± 0.16	0.43 ± 0.22	0.455	0.645
S + RS	Δ TCh	-0.32 ± 0.07	-0.24 ± 0.15	-0.45 ± 0.20	0.350	0.711
	Δ HDL	-0.33 ± 0.07	-0.05 ± 0.05	-0.04 ± 0.05	0.017	0.983
	Δ LDL	-0.14 ± 0.04	-0.12 ± 0.16	-0.52 ± 0.16	2.247	0.145
	Δ TG	-0.31 ± 0.02	-0.14 ± 0.45	0.24 ± 0.17	1.447	0.271

[†]One-way ANOVA with repeated measures. Values in a row with different superscript letters are significantly different. p < 0.05, Bonferroni post-hoc analysis.

5.2.27 Gender differences in lipids

At baseline, females had significantly higher HDL and lower TG compared with males (Table 5.22). During the study, lipids were not affected differently between genders, however while S + RS did not affect HDL in males, there was a significantly different decrease in females. There was also a trend for greater mean decreases in TCh and LDL with S + YP in females.

Table 5.22 Baseline lipids and lipid changes with each dietary period for females and males.

		female	male	p [†]
Week 0	TCh	6.76 ± 0.29	6.57 ± 0.21	0.593
	HDL	1.42 ± 0.08	1.14 ± 0.07	0.015
	LDL	4.58 ± 0.28	4.41 ± 0.25	0.661
	TG	1.66 ± 0.17	2.23 ± 0.19	0.039
S + YC	Δ TCh	-0.06 ± 0.13	-0.16 ± 0.18	0.712
	Δ HDL	-0.01 ± 0.03	-0.01 ± 0.04	0.986
	Δ LDL	-0.27 ± 0.12	-0.06 ± 0.15	0.354
	Δ TG	0.45 ± 0.17	0.06 ± 0.34	0.391
S + YP	Δ TCh	-0.58 ± 0.26	-0.14 ± 0.13	0.119
	Δ HDL	-0.08 ± 0.12	-0.10 ± 0.04	0.851
	Δ LDL	-0.35 ± 0.16	-0.04 ± 0.13	0.153
	Δ TG	0.07 ± 0.29	0.01 ± 0.17	0.853
SC	Δ TCh	-0.22 ± 0.14	-0.01 ± 0.09	0.213
	Δ HDL	-0.05 ± 0.06	0.00 ± 0.04	0.511
	Δ LDL	-0.22 ± 0.15	-0.21 ± 0.11	0.952
	Δ TG	0.11 ± 0.21	0.41 ± 0.29	0.456
S + RS	Δ TCh	-0.42 ± 0.16	-0.32 ± 0.15	0.656
	Δ HDL	-0.11 ± 0.05	0.01 ± 0.03	0.041
	Δ LDL	-0.39 ± 0.12	-0.27 ± 0.15	0.542
	Δ TG	0.20 ± 0.16	-0.13 ± 0.22	0.275

[†]Student's unpaired t-test.

5.3 DISCUSSION

5.3.1 Plasma and urinary isoflavone levels following baseline soy meals

Plasma concentrations of daidzein and genistein were significantly increased 8 h after the test soy meals prior to daily soy intake and still significantly elevated at 24 h. Although this limited sampling regime did not allow for a complete profile of plasma bioavailability, the 8 h and 24 h samples were timed after each test soy meal to allow standardised comparisons of bioavailability. After a single intake of soy in Chapter 3, daidzein and genistein reach their peak plasma concentration between 7 and 9 h, confirming previous reports (King and Bursill, 1998; Setchell and Cassidy, 1999; Setchell *et al.*, 2003b), thus the 8 h plasma sample was expected to be representative of a level of daidzein and genistein close to peak concentration and the 24 h plasma sample representative of the lowest plasma concentrations of daidzein and genistein during a dietary period of daily soy intake.

After the test soy meal that provided a total of 38 mg daidzein and 67 mg genistein (mean intake 0.48 ± 0.01 and 0.84 ± 0.02 mg/kg body mass respectively), plasma concentrations at 8 h and 24 h post-meal were 177 ± 11.4 ng/mL and 41.5 ± 5.6 ng/mL daidzein, respectively and 409 ± 27.1 ng/mL and 132 ± 10.9 ng/mL genistein respectively. Other studies that have used similar isoflavone doses have reported similar subsequent levels of plasma isoflavones. Xu and co-authors (1994) reported 201 ng/mL and 10 ng/mL daidzein at 6.5 h and 24 h, respectively after an intake of soy milk powder providing 0.39 mg daidzein/kg body mass and 581 ng/mL and 70 ng/mL genistein at 6.5 h and 24 h respectively after an intake of soy milk powder providing 0.88 mg genistein/kg body mass. Although these plasma levels at 6.5 h after intake are greater than those reported in the current study at 8 h after soy intake, the levels 24 h post-meal are less, suggesting faster pharmacokinetics when consuming soy in a liquid matrix (King and Bursill, 1998). Following the test soy meal, the plasma genistein concentration was greater than that of daidzein, a finding consistent with other soy

isoflavone bioavailability studies (King and Bursill, 1998; Setchell *et al.*, 2003b). In addition, the mean ratio of daidzein: genistein decreased significantly between 8 and 24 h post-meal (from 0.47 ± 0.02 to 0.33 ± 0.04), suggesting that daidzein has a faster clearance, as previously reported (Setchell *et al.*, 2003a; Shelnutt *et al.*, 2002), or increased metabolism compared with genistein.

The significantly elevated plasma levels of daidzein and genistein 24 h after the test soy meal is important for interpretation of the results of each 5-week dietary period. Although the quantities of soy milk and cereal for daily intake during each dietary period were identical to those consumed for each test soy meal, they were not always consumed in total as a single breakfast serve. However, the 0 h plasma sample in week 5 was taken after a 12 h fast and as none of the subjects consumed both the soy cereal and soy milk with their evening meal (data from end of study questionnaire, Appendix 5.1), these plasma isoflavone levels should have been fairly comparable with the plasma levels 24 h after the test soy meal unless there were additional effects of the regime of soy intake on isoflavone bioavailability (daily intake compared with a single meal).

Urinary excretion during 24 h after the baseline test soy meal was 11.2 ± 0.59 mg daidzein and 4.4 ± 0.49 mg genistein and 24 - 48 h post-meal was 4.0 ± 0.56 mg daidzein and 5.0 ± 0.77 mg genistein. These represent total 48 h urinary recoveries of $40.3 \pm 2.5\%$ daidzein and $14.0 \pm 1.5\%$ genistein. While most of this recovery of daidzein was due to excretion during 0 – 24 h ($29.5 \pm 1.5\%$) compared with 24 – 48 h ($10.5 \pm 1.5\%$), the excretion of genistein was similar between the first and second 24 h after intake ($6.5 \pm 0.73\%$ and $7.5 \pm 1.1\%$, respectively). Although not necessarily reflected in the mean excretion values, the mean ratio of daidzein: genistein in urine was 5.9 ± 0.82 (0 – 24 h) and 2.0 ± 0.33 (24 – 48 h) in contrast to the greater concentration of genistein compared with daidzein in plasma. Therefore, similar to the reduced plasma concentration of daidzein relative to genistein 24 h post-meal indicating earlier removal from plasma, daidzein was also excreted in urine more quickly. In the first 24 h post-meal, approximately 75% of the total daidzein recovered was excreted, while only

approximately 50% of the total genistein recovered was excreted. Most studies report higher urinary levels and recovery of daidzein compared with genistein (Karr *et al.*, 1997; Lampe *et al.*, 1998; Lu *et al.*, 1995; Lu *et al.*, 1996; Tew *et al.*, 1996). After intake of soy milk powder providing 0.39 mg daidzein and 0.88 mg genistein/kg body weight in the current study, 24 h urinary recoveries were 20% daidzein and 10% genistein, similar to those reported here (Setchell *et al.*, 2003a; Xu *et al.*, 1994), however Zhang and co-authors (2003) and King and Bursill, (1998) reported recoveries of approximately 60% daidzein and 20% genistein after intake of soy milk powder and soy flour in cows milk, respectively.

5.3.2 Probiotic effects on soy isoflavone bioavailability

The combination of daily intake of probiotic yoghurt and soy foods did not significantly alter plasma or urinary isoflavone levels compared with intake of soy foods and control yoghurt. The 0 h plasma daidzein and genistein levels were significantly increased after 5 weeks with both dietary treatments (S + YC and S + YP), but there were no significant changes in the plasma isoflavone concentration after the test soy meal (Figure 5.2). There was a trend for these 0 h levels of daidzein and genistein to be higher after intake of probiotic compared with control yoghurt, which suggests a minor enhancement of circulating plasma isoflavone levels with probiotic treatment, but no effect on isoflavone bioavailability resulting from a single soy meal. Both treatments also resulted in the 0 h plasma and daidzein levels at week 5 being higher than those 24 h after the initial test soy meal, which was significant for S + YP. This implies an increased accumulation of plasma isoflavones after 5 weeks of daily soy intake, which was significant when probiotic yoghurt was concurrently consumed. Although there were mean positive, but not significant increases in urinary excretion of daidzein and genistein with both 5-week periods, suggesting a modest increase in isoflavone bioavailability with 5 weeks of daily soy consumption, there were no significant differences between treatments. Overall, although there were no significant effects of probiotic intake on soy isoflavone bioavailability, some results suggested modest increases in plasma isoflavone levels.

It was hypothesized that oral administration of probiotic bacteria with enzyme activity necessary for isoflavone absorption and metabolism may enhance these processes and result in increased isoflavone bioavailability. A lack of a significant effect may indicate either that the levels of probiotic bacteria provided were insufficient to affect gut microfloral balance or to influence isoflavone absorption or that any gut microfloral changes produced did not further affect isoflavone absorption. For health benefits, a minimum daily dose of $10^8 - 10^9$ viable cells of probiotic bacteria is recommended (Chen *et al.*, 1999; Gomes and Malcata, 1999; Shimakawa *et al.*, 2003; Shin *et al.*, 2000). The yoghurt in this study contained 10^8 colony forming units (CFU)/100 g daily serve of each of *Lactobacillus acidophilus*, *Bifidobacterium bifidus* and *Lactobacillus GG*, which should have been sufficient to affect the microfloral balance of the gastrointestinal tract.

Oral administration of probiotic bacteria, including those incorporated in the yoghurt used in this study, results in increased levels of these bacteria in the human gastrointestinal tract (Cummings and Macfarlane, 1997; Dunne *et al.*, 2001; Rolfe, 2000). Lactobacilli and bifidobacteria species are able to adhere to intestinal mucosa and readily colonise the human gastrointestinal tract (Bezkorovainy, 2001; Crittenden *et al.*, 2001; Dunne *et al.*, 2001; Gorbach and Goldin, 1992; Kailasapathy *et al.*, 2000; Rolfe, 2000) where they can exert probiotic effects in both the small and large intestine (Bezkorovainy, 2001; Hove *et al.*, 1999). Furthermore, bifidobacteria (Bartram *et al.*, 1994; Chen *et al.*, 1999; Cummings and Macfarlane, 1997; Hove *et al.*, 1999), *Lactobacillus GG* (Guerin-Danan *et al.*, 1998; Hove *et al.*, 1999) and *Lactobacillus acidophilus* (Dunne *et al.*, 2001) have also been identified in faeces after probiotic intake.

Faecal bacterial activity is also affected by probiotic administration, although the relevance of these effects in relation to endogenous gastrointestinal activity has not been fully established. Lactobacilli bacteria administration has been shown to decrease fecal β -glucuronidase activity (Gorbach and Goldin, 1992; Ling *et al.*, 1994) and a 3 week intake of fermented milk containing *Lactobacillus acidophilus* and *Bifidobacterium bifidus* significantly increased fecal β -glucosidase activity but resulted in no change in β -glucuronidase activity (Marteau *et al.*, 1990). This lack of effect on β -glucuronidase is

consistent with *Lactobacillus* and *Bifidobacterium* species having the lowest activity of this enzyme (Rowland, 1997). Thus, although it seems likely that increased levels of lactobacilli and bifidobacteria would increase intestinal β -glucosidase activity and possibly enhance the initial hydrolysis of isoflavone glycosides, the endogenous levels of these bacteria in subjects in the current study may have already been sufficient for the isoflavones provided from this diet. Conversely, this may also indicate that an increase in β -glucuronidase activity, necessary to re-release isoflavone aglycones after glucuronidation, may be more important in isoflavone bioavailability, but may not have been increased with the current probiotic intake.

The only research that reports the effects of a combination of probiotic bacteria and soy on isoflavone bioavailability was in a recent randomised crossover trial with 40 postmenopausal women (Nettleton *et al.*, 2004). The addition of 10^9 CFU *Lactobacillus acidophilus* and *Bifidobacterium longum* (capsule form) to a daily intake of soy protein isolate providing a mean intake of 15 mg daidzein, 25 mg genistein and 4 mg glycitein for 6 weeks did not alter plasma isoflavone concentrations of daidzein, genistein, equol or ODMA. Plasma samples were taken in the morning after an overnight fast, thus would be similar to the 0 h samples in week 5 in our study and the mean levels of daidzein and genistein were 60 ng/mL and 20 ng/mL respectively. Both plasma and dietary isoflavones are less than half of those reported in our study, hence the relation between intake and plasma absorption are comparable between the two studies. Thus, soy isoflavone bioavailability does not seem to be affected by concurrent probiotic intake as either a supplement (Nettleton *et al.*, 2004) or a dietary component (current study). In addition, although Nettleton and co-authors (2004) did not find any probiotic effects despite a reported increase in fecal bifidobacteria levels, the probiotic capsules also provided 15 – 30 mg/day of fructooligosaccharide, which by virtue of its prebiotic activity has been shown to significantly increase fecal bifidobacteria counts after only 7 days of intake of 10 g/d (Bouhnik *et al.*, 1999).

The yoghurt used in the current study was provided fresh to subjects every 3 weeks so as to minimize any variation in bacteria levels related to storage, however it was reported as

having a refrigerated shelf life, during which levels of each bacteria remained above 10^8 CFU/100 g, of 30 days. Numbers of live bacteria in yoghurt have been reported to decline by an order of magnitude during the three weeks prior to the expiration date, although were still maintained above viable levels until one week past expiration (Shin *et al.*, 2000). However, different absolute amounts of probiotic bacteria may translate to varying endogenous effects and in the current study as subjects collected their fresh batches at different times, there may have been inter-individual variability in the levels of bacteria ingested at equivalent times during the study. In addition, it is difficult to isolate the effects of specific strains as many probiotic preparations may contain multiple bacterial strains (Klaenhammer, 2000; Rolfe, 2000) and there are also discrepancies between the reported quantities in commercially available products, including two Australian studies (Marteau *et al.*, 1990; Micanel *et al.*, 1997; Shah *et al.*, 1995; Shin *et al.*, 2000).

5.3.3 Prebiotic effects (resistant starch) on soy isoflavone bioavailability

For the prebiotic cohort, plasma or urinary isoflavones were not affected significantly differently when resistant starch was consumed concurrently with the control soy diet; both dietary periods (SC and S + RS) significantly increased plasma isoflavone levels (Figure 5.7). However, plasma isoflavone levels 24 h post-meal were highest after 5 weeks S + RS, indicating this treatment may prolong plasma isoflavone clearance following a single soy meal, without an effect on circulation levels during daily soy + RS intake. For six subjects, S + RS appeared to increase daidzein relative to genistein in either plasma, urine or both, which was evident as elevated plasma and urinary daidzein: genistein ratios and a lack of correlation between daidzein and genistein levels of plasma and of urine after 5 weeks RS intake (Table 5.7). Of these subjects, four were eql(+) and 2 were eql(o), which may indicate that subjects with a metabolic capacity to produce equol may have a microflora more amenable to prebiotic effects of RS, particularly in relation to increased daidzein production. Indeed, this ability for increased daidzein absorption and excretion relative to genistein may be a determining factor of equol

production which has been suggested to be dependent in the first instance on initial daidzein levels (Tsangalis *et al.*, 2002), however it is not known if competition exists between daidzein and genistein in processes involved in their bioavailability. In addition, increased urinary daidzein excretion after 2 weeks of daily RS intake was also reported in Chapter 3, in which the largest increase in urinary daidzein excretion was observed for a subject who consistently excreted large amounts of equol.

Although no other studies have reported a dietary combination of RS and soy in humans with which the results reported here can be compared, the prebiotic activity of RS has been demonstrated. Resistant starch can specifically stimulate the growth of bifidobacteria in the colon (Brown *et al.*, 1999; Wang *et al.*, 2002) as this is one of only a few species with the capacity to degrade resistant starch (Wang *et al.*, 2002). In addition, dietary supplementation with RS has been shown to increase fecal *Eubacterium* species in humans (Schwartz *et al.*, 2002) and to increase cecal levels of lactobacilli and bifidobacteria in human flora-associated rats (Silvi *et al.*, 1999). Both bifidobacteria and lactobacilli possess high β -glucosidase activity (Marteau *et al.*, 1990; Silvi *et al.*, 1999) and also are the predominate native microflora in the duodenum and jejunum where most of the initial isoflavone hydrolysis is likely to occur (Turner *et al.*, 2003). Bifidobacteria can hydrolyse daidzin and genistin from soy milk to their respective aglycones *in vitro* and further metabolise daidzein to equol (Tsangalis *et al.*, 2002); in human faecal samples bifidobacteria can also metabolise daidzein (Chang and Nair, 1995; Kim *et al.*, 1998).

In vitro, there are strong associations between β -glucosidase activity, bifidobacteria and isoflavone metabolism (Marteau *et al.*, 1990; Tsangalis *et al.*, 2002). Accordingly, β -glucosidase activity in the cecum increased in human flora-associated rats fed RS (Silvi *et al.*, 1999). However, 4 weeks of intake of 55 g RS/day in humans decreased fecal β -glucosidase activity (Hylla *et al.*, 1998). Although the effects of RS and other prebiotics on microfloral balance are well-established, resulting changes in enzymatic activity are less understood. While some studies include fecal bacterial enzyme measures, these may

not be relevant to changes that occur in the small intestine, particularly in relation to isoflavone bioavailability.

The total substrate availability in the human adult colon is between 20 and 60 g of carbohydrate per day (Salminen *et al.*, 1998). Whilst no specific recommendations exist for resistant starch intake, 20 g/day has been suggested as beneficial in relation to colonic health, and this amount can induce bacterial changes (Baghurst *et al.*, 1996). Gastrointestinal effects have been reported for RS intake at daily levels of intake between 15 and 55 g after 2 – 4 weeks (Cummings *et al.*, 1996; Hylla *et al.*, 1998; Phillips *et al.*, 1995; Schwiertz *et al.*, 2002; Topping and Clifton, 2001), although Jenkins and others (1999b) reported no changes after 2 weeks in the output of total bacteria, bifidobacteria, fusobacteria or bacteroides with dietary addition of 21.5 g/day of high amylose corn starch. Similarly, seven days intake of 10 g FOS/day significantly increased fecal bifidobacteria (Bouhnik *et al.*, 1999) and doses of between 5 and 20 g per day caused dose-related increases in breath hydrogen after 12 days (Cummings *et al.*, 2001). There are differences in fermentation between RS and FOS, with FOS fermented rapidly and completely, whereas RS has a slow, prolonged fermentation of only about 70 – 80% (Alles *et al.*, 1997). However, they have similar prebiotic action and it can be assumed that the daily intake of RS in the current study should have been sufficient to produced gut microfloral modifications. However, the lack of subsequent effect on isoflavone bioavailability suggests that these changes were not sufficient for or specific to this process in the subjects in this study.

In Chapter 3 it was reported that urinary daidzein and equol production after a single soy meal increased with daily RS intake. From this result, it was hypothesized that chronic consumption of RS and soy would enhance isoflavone bioavailability. Although in the current study there was a suggestion of increased daidzein production in some subjects, possibly related to a particular gut microfloral profile associated with the metabolism of daidzein to equol as also found in Chapter 3, there were no significant effects of addition of this prebiotic to a soy diet. However an interesting finding in the current study that may highlight the mechanism of RS action in regard to isoflavone bioavailability was a

trend for plasma daidzein and genistein levels 24 h after the test soy meal (but not at 0 h) to be highest after 5 weeks of daily soy + RS intake (Table 5.5). Subjects were encouraged to consume their soy products at separate times to the RS-containing bread based on the results of acute RS intake reported in Chapter 3. The increased plasma isoflavone levels 24 h after the test soy meal but not 0 h after 5 weeks intake of S + RS suggest that when soy and RS are consumed in the same day, isoflavone bioavailability is not increased, but when resistant starch is consumed the day prior to soy intake, isoflavone bioavailability may be enhanced. RS perhaps has local effects in the gut that may inhibit, or at least not increase, the absorption of soy isoflavones but may produce bacterial changes that are appropriate to increase soy isoflavone bioavailability more than 24 h later.

5.3.4 Probiotic and prebiotic effects on equol production

There was clear distinction between subjects in the current study in regards to equol-producing ability in the frequency of equol occurrence rather than the absolute amount in plasma or urine and resulted in tertiles of subjects classified according to their plasma or urinary incidence of equol. Based on these classifications, 39% of subjects in this study (42% of males and 33% of females) were equol-producers which agrees with the incidence reported in many other studies (Lampe *et al.*, 1998; Lampe *et al.*, 2001; Slavin *et al.*, 1998). Most other studies however, have defined an equol-producer based on minimum plasma or urine equol levels (Lampe *et al.*, 1998; Rowland, 1999b) and as such, the method used for classification in the current study differs to those previously reported.

All four soy dietary periods increased 0 h plasma equol levels which implies that 5 weeks of daily soy intake (with or without a probiotic or prebiotic) was sufficient to increase plasma equol in those who are predisposed to this conversion. However, the concurrent consumption of either a probiotic or prebiotic with soy did not affect the number of people in whom equol was present in their plasma or urine, nor did it significantly affect

equol levels in those people who originally produced this daidzein metabolite. Therefore it seems these dietary changes were unable to induce this conversion. In addition, there was a trend of increased plasma equol after 5 weeks of soy + probiotic yoghurt intake compared with the other three dietary periods (Table 5.11). Similar to the trend described previously for increased circulating plasma levels of daidzein and genistein after a combination of soy and probiotic intake, the same trend of increased plasma equol may suggest a potential for orally ingested probiotic bacteria to increase isoflavone intestinal absorption and metabolism. This is most likely related to the lactobacilli and bifidobacteria in the yoghurt resulting in increased levels of these probiotic bacteria (Cummings and Macfarlane, 1997; Dunne *et al.*, 2001; Rolfe, 2000) and possibly increasing β -glucosidase activity (Marteau *et al.*, 1990) in the gastrointestinal tract. β -glucosidases play a key role in the hydrolysis of isoflavone glycosides and increased further metabolism to equol by probiotic intake may be dependent on either increased initial production or enhanced secondary conversion of daidzein.

In the current study, although there was a trend for increased plasma equol with soy intake, there was no similar trend in the small numbers of subjects who excreted equol in their urine. A similar study that examined the combined effects of probiotic and soy intake reported no changes in plasma or urinary equol or the number of equol producers (Nettleton *et al.*, 2004). Similarly, when soy protein powder was consumed with bran cereal providing 16 g dietary fibre per day for one menstrual cycle, no effects were observed on equol-excretor status or 24 h urinary equol excretion (Lampe *et al.*, 2001). Thus, there has been no success in altering equol-producing status in previous dietary studies and at present, gastrointestinal factors determining the production of equol have not been established. However, recent studies examining the bacterial composition of human faecal samples of individuals who have shown the capacity to produce equol, have attempted to elucidate the bacteria involved in daidzein metabolism to equol. A number of bacteria have been identified as playing a role in daidzein metabolism to equol including enterococci, lactobacilli, bifidobacteria, streptococci, ruminococci and bacteroides (Decroos *et al.*, 2005; Tsangalis *et al.*, 2002; Ueno and Uchiyama, 2001), however more than a single species is involved. Based on antibiotic effects on equol

production in human faecal samples, Atkinson and co-authors (2004) suggested that the conversion of daidzein to dihydrodaidzein and of dihydrodaidzein to equol might be carried out by different bacteria and that the bacteria involved may differ between individuals. Further investigation into the gut microflora involved in this metabolism will assist in the establishment of dietary conditions which may be able to affect equol production.

Apart from the specific bacteria involved, modification of other gastrointestinal characteristics may influence equol production. Factors affecting equol production in faecal samples from subjects who are able to produce equol were reported in two recent studies. Atkinson and co-authors (2004) reported that anaerobic conditions were necessary for daidzein conversion to equol and Decroos and co-authors (2005) reported that although equol production was inhibited by addition of FOS, it was increased by the presence of hydrogen as well as propionate and butyrate. These findings are somewhat contradictory, since hydrogen gas is a major fermentation product of carbohydrates, including FOS, in the gut (Decroos *et al.*, 2005). However as the major bacteria induced by FOS intake is bifidobacteria, which were not apparently responsible for faecal equol production in that study, perhaps this prebiotic resulted in other changes in equol-producing bacterial populations which may have been a more significant effect than any increases in hydrogen (Decroos *et al.*, 2005). An analogous situation may explain the lack of increase in equol production in the current study with RS intake. Although the major short chain fatty acid products of RS intestinal fermentation are propionate and butyrate (Brown *et al.*, 1998; Rowland, 1999a; Topping and Clifton, 2001), which increased equol production (Decroos *et al.*, 2005), the prebiotic effects on gut microflora may not have been advantageous in relation to equol production. However, diet was shown to affect faecal equol production in a study by Rafii and colleagues (2003), in which increased consumption of fruits and vegetables resulted in greater *in vitro* faecal equol production in two subjects. Interestingly, onions and leek are a source of FOS (Alles *et al.*, 1997), which may have contributed to this effect.

It should be noted that the faecal samples analysed in the study reporting on identification of faecal bacteria involved in the metabolism of daidzein to equol (Decroos *et al.*, 2005) were provided from the current study. This opportunity arose after initial study design, and allowed for some comparison between faecal isoflavone metabolic abilities with plasma and urinary isoflavone levels, even in this limited number of samples. The faecal isoflavone metabolic analyses (Decroos *et al.*, 2005) and the plasma and urinary isoflavone analyses reported here were conducted independently and without prior knowledge of the other finding. Interestingly, the faecal sample from subject PB06 in the current study, who had large plasma ODMA levels (Table 5.12), produced ODMA as reported as Sample 2 by Decroos and colleagues (2005). In addition, the faecal sample identified as Sample 4 by Decroos and colleagues (2005) and found to produce equol was from a subject classified as equol-positive in the current study. However, while Sample 3 did not produce equol in culture (Decroos *et al.*, 2005), this subject had been identified as equol-positive in the current study, although they only had plasma equol after 2 of the 4 test soy meals. This highlights variability in equol-producing capacity even within an individual, but also confirms a relation between *in vitro* faecal production and plasma and urinary levels of equol and ODMA.

Gastrointestinal conditions related to equol production may be inherent and not modifiable through dietary change. Akaza and co-authors (2004) reported a higher proportion of equol producers among Japanese and Korean men (46 and 59%, respectively) compared with American men (14%). Although this may be related to diet and gut microflora, genetic background may also play an important role. While some investigators have reported consistent equol-producing ability within subjects when re-tested up to 10 months apart (Hendrich *et al.*, 1998; Zhang *et al.*, 1999), others have concluded equol-producing ability of an individual after a single soy intake. Nonetheless, the results of the current study suggest that this may not be a reliable procedure as inconsistent equol production was found within the same subjects over time. Rafii and colleagues (2003) also reported that faecal samples obtained from the same individuals at different times varied in the metabolism of daidzein for some subjects, a similar trend to that observed in plasma and urinary equol in the current study.

This may indicate that while genetics may play some part in determining who will produce equol after soy intake, other characteristics such as habitual or acute dietary intake may enhance or reduce equol this capacity on different occasions in these individuals.

ODMA was present in only two subjects in this study in contrast to the reportedly common occurrence of this daidzein metabolite after soy intake (Atkinson *et al.*, 2004; Decroos *et al.*, 2005). The only subject who had consistent levels of plasma ODMA did not produce equol at any time during the study, suggesting that these pathways of daidzein metabolism are inversely related or mutually exclusive, in agreement with other findings (Kelly *et al.*, 1993; Kelly *et al.*, 1995; Slavin *et al.*, 1998). In contrast, another subject showed the metabolism of daidzein in a single plasma sample during the study and this sample contained both equol and ODMA. Factors determining the pathways of daidzein metabolism to either equol or ODMA are unknown and further highlight individual variability. Rafii and co-authors (2003) observed *in vitro* production of both equol and ODMA by the microflora of the same individual and Decroos and colleagues (2005) suggested that equol and ODMA are formed by different bacteria, but that these species can co-exist. Atkinson and co-authors (2004) suggested that in the presence of equol-producing bacteria, the conversion of daidzein to ODMA is impaired, either because the production of equol inhibits growth of ODMA-producing bacteria or that there may be competition for the substrates (Atkinson *et al.*, 2004). Thus, the current study and others show large variation between individuals in daidzein metabolism. Although it was expected that probiotic and/or prebiotic intake could affect daidzein metabolism to equol or ODMA, a definitive relationship was not found in the current study.

5.3.5 Comparison between probiotic and prebiotic effects on isoflavone bioavailability

Although there were no significant differences in isoflavone bioavailability when soy was consumed concurrently with either a probiotic or prebiotic with soy, there was a trend for a greater increase in 0 h plasma daidzein and genistein levels with probiotic treatment and in 8 h and 24 h levels with prebiotic treatment. There also appeared to be a particular effect of S + RS to increase plasma and urinary daidzein, evident in the daidzein: genistein ratios and correlation analysis. For all other treatments, the daidzein: genistein ratio was highest at 8 h and lowest at 24 h, however there was no reduction in the ratio between these times for S + RS. In addition, after 5 weeks S + RS, there was a lack of correlation between plasma daidzein and genistein at 0 h, due to a noticeably large increase in daidzein in 4 subjects after 5 weeks of daily S + RS intake. Similarly in urine, there was a trend for the highest ratio of daidzein: genistein to occur after 5 weeks S + RS, indicating that this treatment increased urinary excretion of daidzein more than that of genistein and more than the other treatments. There was also a trend for increased plasma equol with probiotic treatment.

It is difficult to compare the probiotic and prebiotic treatments given the lack of significant effects with either. However, any differences were expected to be due to the duration of the bacterial effects in the gut, which differs between these two dietary components. While the colonisation of probiotic bacteria is only temporary, generally lasting for as long as the bacteria is ingested (Chen *et al.*, 1999; Cummings and Macfarlane, 1997; Hove *et al.*, 1999), prebiotic effects including those of RS last longer than the duration of ingestion due to an increase in substrate for bacterial growth. Schwartz and co-authors (2002) reported that gut microfloral effects of RS can persist even 2 weeks after discontinuation of intake. It was also speculated in the current study that RS may have had more of an effect on isoflavone bioavailability than probiotic intake due to differences in duration of effect and bacterial influences. While probiotic intake generally has more of an influence on lactobacilli, prebiotic intake more specifically affects bifidobacteria (Brown *et al.*, 1999; Rowland, 1999a; Silvi *et al.*,

1999). As β -glucosidase activity is more related to bifidobacteria, it was expected that RS intake would have had more of an effect on isoflavone bioavailability due to effects on this enzyme (Marteau *et al.*, 1990).

There may however be a limit as to how much the endogenous gut microfloral levels can be increased by probiotic or prebiotic intake and it has been suggested that the stimulation of growth of bifidobacteria depends on the initial levels (Roberfroid, 1998). Thus, in the current study, subjects may have had sufficient levels of these gut microflora such that probiotic or prebiotic intake did not have additive effects. Alternatively, the large variability in results may have been due to greater effects in some individuals with lower levels of these bacteria initially. One study reported a significant increase in faecal levels of bifidobacteria and decrease in enterococci with ingestion of fermented milk containing *Lactobacillus* species but no overall change in the species composition of these bacterial species per individual (Saito *et al.*, 2002). This suggests that faecal microfloral composition is stable and not affected by foods that can influence gut microflora.

The potential of a combination of resistant starch and probiotic cultures for synergistic gut microfloral effects in relation to isoflavone bioavailability may be more beneficial as it would increase both the substrate for bacterial growth as well as these bacterial populations. This is the concept underpinning the use of synbiotics. A synbiotic (in which both a probiotic and prebiotic are combined in a single product) can potentially improve the survival and establishment of ingested live bacteria in addition to stimulating the growth and/or activity of the probiotic bacteria and of specific resident probiotic bacteria (Roberfroid, 1998). Synbiotics have been proposed as having greater health benefits than probiotics or prebiotic alone (Bezkorovainy, 2001; Brady *et al.*, 2000; Brown *et al.*, 1999; Kailasapathy *et al.*, 2000). In particular, soybean oligosaccharides have been suggested as having prebiotic activity as they are fermented by colonic microflora possessing α -galactosidase activity, including bifidobacteria (Boever *et al.*, 2000; Gibson and Fuller, 2000) and soygerm powder has been shown to increase the *in vitro* survival of *Lactobacillus reuteri* subjected to acidic conditions (De Boever *et al.*,

2001). There is the potential for the combination of the probiotic and prebiotic components specifically used in the current study as Crittendon and colleagues (2001) isolated a *Bifidobacterium* strain for inclusion with RS in a synbiotic yoghurt.

5.3.6 Effects of gender on isoflavone metabolism

After the initial test soy meal, females had significantly higher plasma daidzein than men 8 and 24 h post-meal and there was a trend for higher plasma genistein 8 h post-meal. In contrast, there were no gender differences in urinary isoflavone excretion. Also throughout the study there was a trend for greater mean plasma levels of isoflavones in females than men but no differences in urinary isoflavone levels. However, there was a significant disparity between genders for body mass, with females weighing significantly less than males and correspondingly, this equated to differences in the mass-specific isoflavone intake. The mean intake for females was 0.52 ± 0.03 mg/kg daidzein and 0.92 ± 0.05 mg/kg genistein and for males was 0.45 ± 0.01 mg/kg daidzein and 0.79 ± 0.02 mg/kg genistein and may partly account for higher plasma isoflavone levels in females. However as females did not excrete greater amounts of isoflavones in their urine, this may indicate greater retention of the isoflavones in the body. This may be associated with sequestration of isoflavones in tissues or fat, especially in organs with a high density of estrogen receptors (Maubach *et al.*, 2003). Conversely, there may be differences between genders in the kinetics of isoflavone absorption, metabolism or excretion. Lampe and colleagues (1998) reported gender differences in colonic function, including in gastrointestinal transit time, faecal bulk, bile acid excretion and in responses to the amount and type of dietary fibre fed, which may contribute to the effects observed in the current study. There were no differences between the genders in their response to probiotic or prebiotic treatment though, however Silvi and co-authors (1999) reported significant differences between male and female rats in bacterial counts after RS administration.

For females in the current study, there were further correlations between age and plasma levels of isoflavones, providing more evidence for a role of estrogen receptors or endogenous estrogen activity in influencing isoflavone bioavailability. The number of years since menopause would affect estrogen profile and receptor availability and could potentially affect isoflavone absorption and retention, either in relation to estrogen receptor densities in different tissues or circulating estrogen levels; however, this has not been examined. Frankenfeld and co-authors (2003) also reported that older age was associated with higher plasma daidzein and genistein concentrations in Western postmenopausal women aged between 50 and 79 years. These authors found an association between each increasing year of age and an increase of 0.7 mg/week in isoflavone intake, however the average isoflavone intake per week (8 mg genistein and 6 mg genistein) and the mean plasma levels were quite low (12 nmol/L daidzein and 7 nmol/L genistein). Other factors may have contributed to the higher plasma concentrations in women, including altered renal clearance of isoflavones (Frankenfeld *et al.*, 2003). In addition, aging has been associated with decreased colonic motility (Hosoda *et al.*, 1992) and a significant reduction in anaerobic bacteria (Mitsuoka, 1992), specifically bifidobacteria (Gomes and Malcata, 1999). However, if these factors were having an influence on isoflavone bioavailability in the current study, age-related effects in men should have also been apparent. Perhaps in combination with additional influences of endogenous hormone levels in females, differences in isoflavone bioavailability with age may be exemplified.

5.3.7 Inter-individual variability in isoflavone bioavailability

There was large variability between individuals in isoflavone bioavailability (plasma and urinary isoflavones) and in the effects of probiotic and prebiotic treatments. Although there was a large range of intake of daidzein and genistein between subjects in the current study when calculated per kg body mass, the largest mean change in body mass over any of the weeks was 0.3 kg, thus per individual, isoflavone intake would have remained constant. Thus, although this may have contributed to some variation, it would

not have influenced the effects of any dietary treatment. The particular effect of S + RS to increase daidzein bioavailability only in some subjects may be due to the wide individual variation that is known to occur in response to fermentation of prebiotics (Cummings *et al.*, 2001). Furthermore, differences between the gut microflora composition of subjects may also have determined subsequent effects of probiotic or prebiotic intake.

There was high correlation between plasma daidzein and genistein at 8 h post-meal after all 5-week dietary periods. In addition, plasma daidzein and genistein were also correlated at 0 h after S + YC, S + YP and SC, and after 24 h only after S + YC and S + RS (Table 5.7). This significant correlation at 8 h, close to the time when daidzein and genistein reach their peak plasma levels, may indicate similar absorption kinetics for these isoflavones per individual, while reduced correlation at 24 h post-meal suggests that the clearance from the plasma may be more variable between subjects and/or different for the two isoflavones. Correlation of daidzein and genistein in urine samples was also significant, which suggests that although there was large inter-individual variation in urinary excretion of isoflavones, an individual seems able to excrete daidzein and genistein similarly. For all treatments excepting the prebiotic (S + RS), the mean plasma D/G ratio was highest at 8 h and lowest at 24 h, which suggests that daidzein may either be metabolised more or cleared faster from the plasma between 8 and 24 h post-meal compared with genistein. However, although the plasma D/G ratio was lowest 24 h post-meal, implying that daidzein was cleared from the plasma faster than genistein, this disparity in plasma clearance rates of daidzein and genistein was not reflected in that of the urine, from which it appears genistein is excreted more quickly.

5.3.8 Effects of soy intake and dietary combination with probiotic and prebiotic consumption on lipids

In subjects who were hyperlipidemic on entry to this study, there were few significant effects of any of the 5-week dietary periods on lipids (5.3.13). Soy + probiotic yoghurt

intake significantly decreased TCh by $4.7 \pm 2.0\%$ (mean change -0.32 ± 0.14 mmol/L) and soy + resistant starch enriched bread intake significantly decreased TCh by $5.5 \pm 1.6\%$ (-0.36 ± 0.10 mmol/L) and LDL by $7.3 \pm 2.2\%$ (-0.32 ± 0.10 mmol/L). The only effect of either control soy treatment was of a significant reduction in LDL with the soy control of the prebiotic cohort of $4.1 \pm 2.1\%$ (-0.21 ± 0.09 mmol/L). It was hypothesised that the control soy treatments would also produce a hypocholesterolemic effect, particularly with the elevated baseline lipids in this study population. This was evident in a trend for a reduction in mean TCh with both soy control periods, but these were not significant effects. In Chapter 4 it was found that soy consumption resulted in more of a lipid-lowering effect in subjects with baseline TCh greater than 6.0 mmol/L and in the current study, 24 of the 31 subjects had baseline TCh greater than 6.0 mmol/L at the beginning of the soy dietary periods. Compared with the former study, the soy intake in the current study provided less soy protein but a comparable daily intake of total isoflavones.

During all dietary treatments of the current study, subjects consumed approximately 10 g soy protein and 38 mg daidzein and 68 mg genistein and 4.5 mg glycitein per day. The lack of a reduction in TCh or LDL levels with the soy control treatments may provide evidence for the necessity of a minimum amount of soy protein for lipid-lowering effects. The 1999 health claim for the hypocholesterolemic effect of soy protein (F.D.A., U.S.) was based on a daily intake of 25 g and 3 meta-analyses that have reported overall reductions of approximately 4% or more in total cholesterol have reported mean daily soy protein intakes of between 22.5 and 47 g per day (Anderson *et al.*, 1995; Harland and Carr, 2004; Weggemans and Trautwein, 2003). Thus, the results reported here, with a daily soy protein intake less than those reported in meta-analyses suggests that daily intake of 10 g soy protein is insufficient, even in the presence of a total of 110.5 mg isoflavones for significant hypocholesterolemic effects with soy consumption.

In the meta analysis by (Anderson *et al.*, 1995), only subjects with TCh >6.7 mmol/L had significant lipid lowering benefits. In the present study, there were only 10 subjects

whose TCh was greater than 6.7 mmol/L at the beginning of the control soy dietary period. For these subjects, independent of which cohort they were in, there was a mean significant decrease in TCh of -0.32 ± 0.13 mmol/L ($-4.1 \pm 1.8\%$) from a baseline level of 7.63 ± 0.30 ($p = 0.04$, Student's paired t-test). Thus, similar to that reported in the meta-analysis, subjects in the current study with baseline TCh greater than 6.7 mmol/L experienced a significant lipid-lowering and thus health beneficial effect of soy intake.

Even though neither soy control period resulted in significant reductions in TCh, dietary combination of soy with either probiotic or prebiotic consumption significantly decreased TCh. As there was a trend towards a reduction in TCh with soy control, it appears that the effects of the combination with probiotic or prebiotic intake would have been due to an additive effect rather than solely attributable to either the probiotic or prebiotic component. However, as the study design was not to compare effects of the dietary combination with those of control probiotic or prebiotic treatments without soy, the proportional effect of each component can not be calculated accurately. In the only other study to date to report on the effects of combined soy and probiotic intake on lipids, Greany and co-authors (2004) reported contrasting results to those of the current study. In hypercholesterolemic subjects with baseline TCh between 5.17 and 6.59 mmol/L, 6 weeks of intake of soy protein isolate (providing 25 mg genistein, 15 mg daidzein, 4 mg glycitein + 26 g soy protein per day) significantly reduced TCh and LDL by 5% and 6%, respectively (Greany *et al.*, 2004); however, concurrent intake of 10^9 CFU *Lactobacillus acidophilus* and *Bifidobacterium bifidum* did not result in any further effects. This lack of effect of the combination of soy and probiotic intake may have been because the soy itself had already produced a significant hypocholesterolemic effect which may have been maximal.

However, intake of both probiotics and prebiotics is often associated with lipid benefits in humans. A hypocholesterolemic effect of fermented milk was first observed in Massai tribesmen (Sharper *et al.*, 1963) and since then, much animal evidence has supported this effect (Taylor and Williams, 1998). Human studies have also demonstrated lipid-lowering effects of probiotic although not unequivocally and de Roos and Katan (2000)

concluded in their review that effects of probiotic bacteria on lipids need further investigation. A meta-analysis of 6 studies using a fermented probiotic bacteria product reported significant reductions in TCh and LDL of 6% and 9%, respectively with between 4 and 8 weeks intake, or between 4% and 5%, respectively when an outlying study was removed from analysis, and concluded that this was a clinically important reduction in plasma cholesterol (Agerholm-Larsen *et al.*, 2000a). In addition, the effects of this yoghurt did not differ significantly to those of other fermented yoghurts (Agerholm-Larsen *et al.*, 2000b).

Significant reductions in TCh and LDL of between 4 and 6% and 5 and 10%, respectively have been reported from intake of fermented milk products of between 3 weeks and 3 months duration. These studies had mean baseline levels of between 5.2 and 6.1 mmol/L TCh and between 3.4 and 4.3 mmol/L LDL (Agerbaek *et al.*, 1995; Richelson *et al.*, 1996; Schaafsma *et al.*, 1998). However, another study reported significant reductions in TCh (6%) after 4 weeks intake of milk products fermented by *Bifidobacterium longum* only for subjects with baseline TCh levels greater than 6.2 mmol/L (Xiao *et al.*, 2003) and others have reported no lipid effects with probiotic intake (de Roos *et al.*, 1999; Kiebling *et al.*, 2002). Triglycerides and HDL are largely unaffected by probiotic intake, even in studies that have reported other lipid effects (Agerbaek *et al.*, 1995; Richelson *et al.*, 1996; Schaafsma *et al.*, 1998), however Kiebling and colleagues (2002) reported a significant increase in HDL (0.32 mmol/L) after 6 month intake of milk fermented with *Lactobacillus acidophilus* and *Bifidobacterium longum* in combination with 1% oligofructose. The yoghurt in this study contained 10⁸ colony forming units (CFU)/100 g daily serve of each of *Lactobacillus acidophilus*, *Bifidobacterium bifidus* and *Lactobacillus GG*, which are similar levels compared with other studies examining the effects of probiotic bacteria on lipids (Greany *et al.*, 2004; Xiao *et al.*, 2003).

There is also animal evidence to suggest that prebiotic intake can significantly decrease lipids and TG (Taylor and Williams, 1998), human evidence is lacking in this area. Intake of 21.5 g/d high amylose corn starch for 2 weeks RS was associated with lower

TCh and LDL and higher HDL (Jenkins *et al.*, 1999b), however these authors had previously reported no effect of intake of 28 g RS per day for 2 weeks on serum lipids, though these were healthy subjects including premenopausal women (Jenkins *et al.*, 1998). There may be a particular effect of the combination of prebiotic intake with other lipid-lowering regimes as was observed in the current study. Muir and co-authors (1998) reported significantly lower TCh, HDL and LDL after 3 weeks intake of a Chinese-based diet providing 18 g RS and higher vegetable protein, partly from soy compared with a Western-based diet of 8 g RS and less vegetable protein content. Jenkins and colleagues (1999a) reported that dietary combination including 33 g soy protein and 8 g soluble fibre reduced TCh and LDL by 11 and 13%, respectively and that each of these components was responsible for 50% of the effect. Further, two of the products in Europe marketed for lowering cholesterol, contain a probiotic and a prebiotic (Pereira and Gibson, 2002). In the current study, there was a particular effect observed with RS intake of prolonged lipid-lowering effects even after intake had ceased. In the prebiotic group who consumed S + RS in the first dietary period (Pre-2), TCh was reduced by 0.18 ± 0.07 mmol/L. However, after the 4-week washout period, TCh had been further reduced by 0.15 ± 0.10 mmol/L which may suggest a long-lasting effect of RS intake on lipids. In contrast, no other groups showed a mean decrease in TCh during the washout period.

There have been several suggestions made as to the mechanisms of lipid-lowering by probiotic bacteria mostly related to their effects on bile acids. *In vitro*, probiotic bacteria including *Lactobacillus acidophilus* and *Bifidobacterium bifidus* can assimilate cholesterol (Gomes and Malcata, 1999) and cause deconjugation of bile salts (Pereira and Gibson, 2002). This also suggests that probiotic bacteria may assimilate some of the cholesterol from the diet, making it unavailable for absorption (Pereira and Gibson, 2002; Xiao *et al.*, 2003). Further, probiotic intake has been proposed to increase bile acid excretion which would reduce serum cholesterol because of conversion of the latter to replace the excreted bile acids (Pereira and Gibson, 2002; Sanders, 2000). However, these effects are dependent on the viability of the ingested bacterial strains in the human gastrointestinal tract and their ability to colonise the small intestine where most of

cholesterol absorption takes place (Pereira and Gibson, 2002). In relation to prebiotic actions on lipids, Jenkins and colleagues (1999b) suggested that colonic microbial activity is related to lipid risk factors for CVD and in particular that the production of acetate during carbohydrate fermentation by the microflora may increase, but the production of propionate may decrease serum cholesterol. Interestingly, the major short chain fatty acid products of RS intestinal fermentation are propionate and butyrate (Brown *et al.*, 1998; Rowland, 1999; Topping and Clifton, 2001).

5.3.9 Study design

Plasma and urinary isoflavones following the test soy meals were not significantly different between week 0 and week 9 prior to each corresponding 5-week dietary period, demonstrating that the 4-week washout period during which soy was removed from the diet was sufficient to allow return to baseline after any changes resulting from the 5-week intervention. This also demonstrated that the first 5-week period of daily intake of soy foods had no longer term effects on soy isoflavone bioavailability. Thus, the use of a crossover design with a 4-week washout period was sufficient, although there appeared to be long-term effects of S + RS on lipid levels which may require a longer washout period for use in a crossover study.

5.3.10 Conclusions and future directions

In this study, 5 weeks of daily intake of soy milk and soy cereal increased circulating plasma levels of daidzein and genistein but did not result in enhanced isoflavone bioavailability from a test soy meal. The concurrent consumption of probiotic-containing yoghurt or prebiotic (RS)-containing bread had no additional significant effects on isoflavone bioavailability, however there was a trend for higher circulating levels of daidzein and genistein with probiotic intake and of higher plasma levels 24 h after the test soy meal with prebiotic intake. Soy intake increased equol production in those

subjects with this ability, but neither probiotic nor prebiotic intake increased the number of subjects who were able to produce equol. However, there was a trend towards higher plasma equol after probiotic intake with soy. There were modest non-significant lipid-lowering effects with the soy control dietary treatments and LDL was significantly reduced with the soy control of the prebiotic cohort. However, when subjects were classified according to their baseline TCh levels, there was a significant reduction in TCh (-0.32 ± 0.13 mmol/L, $-4.1 \pm 1.8\%$) with soy in those with baseline TCh greater than 6.7 mmol/L. The combination of soy with a probiotic or a prebiotic resulted in significant reductions in TCh of $-4.7 \pm 2.0\%$ and $-5.5 \pm 1.6\%$, respectively and thus suggests a potential synergistic hypocholesterolemic action between soy and probiotic bacteria. The easy incorporation of RS into bread (Brown, 1996; Hoebler *et al.*, 1999) and the inclusion of probiotic bacteria in a number of different food vehicles may be particularly beneficial in relation to hypercholesterolemia, especially in combination with dietary soy.

Additional findings in this study were of higher plasma daidzein in females than males, possibly related to the significant inverse correlation between plasma daidzein and weight and also of a significant correlation between 24 h plasma daidzein and age in females. This warrants further investigation into the potential tissue distribution of isoflavones after ingestion, particularly in relation to estrogen levels and estrogen receptor distribution in postmenopausal women and may assist in understanding the mechanisms for endogenous actions of isoflavones. In addition, the trends of some increases in isoflavone bioavailability with soy and concurrent probiotic or prebiotic intake suggest a potential for probiotic bacteria to enhance soy isoflavone bioavailability. Further examination is necessary to help elucidate particular bacteria or gastrointestinal species pertinent to this process.

5.4 APPENDIX

Appendix 5.1. Completion of Study Questionnaire (modified for inclusion)

University of Wollongong

PRE- / PROBIOTIC and SOY STUDY – Participation Questionnaire

A. SOY CEREAL

1. How often did you consume the cereal during

The 1st 5-week period? _____

The 2nd 5-week period? _____

2. How did you find the serving size?

3. Did you consume it all at once or split it over the day?

4. At which meal/snack time(s) did you usually consume the cereal, and what food was this in place of?

5. Please rate your like/dislike of the cereal with a cross on the scale below:

STRONG
DISLIKE

No OPINION

STRONG
LIKE

6. Would you continue to eat this cereal:

a. because of it's taste? _____

b. if it was found to provide health benefits? _____

8. Are there any particular features of the cereal that you did or didn't like (eg texture, blandness, heaviness, fibre, sweetness, etc)? Please describe.

9. Did your bowel movements change while consuming this cereal, and was this favourable?

10. Overall, did you feel any other health-related changes while consuming this cereal? _____

B. SOY MILK

1. How many full serves (250mL) of soy milk (i.e. full glass or on full bowl of cereal) did you consume during:

1st 5-week period? _____

2nd 5-week period? _____

2. How did you usually consume your soy milk serve(s) during the day?

3. Which type of milk did you usually consume before the study?

4. Did you continue to drink other milk during the study? _____

If yes, how much per day? _____

5. Please rate your like/dislike of the soy milk with a cross on the scale below:

STRONG

No OPINION

STRONG

DISLIKE

LIKE

6. Would you continue to drink this milk:

c. because of its taste? _____

d. if it was found to provide health benefits? _____

7. Are there any particular features of the milk that you did or didn't like (eg, taste, fullness, texture, sweetness, fat content etc)? Please describe.

8. Would you have preferred a low fat variety? _____

9. Would you choose this “calcium enriched” variety? _____

10. Did your bowel habits change while consuming this milk, and was this favourable?

11. Overall, did you notice any other health changes that you feel may have been attributed to by the milk?

C. YOGHURT (only answer if you were given yoghurt during the study)

1. How often did you consume the yoghurt during:

1st 5-week period? _____

2nd 5-week period? _____

2. Did you notice a difference in taste or effect of the yoghurt between the 2 periods?
If so, which did you prefer?

3. When did you usually consume your yoghurt during the day?

4. What did you normally eat your yoghurt with, or add to it?

5. How did you find the serving size?

6. Did you normally consume yoghurt before the study? If yes, how often, and what brand, flavour and fat content was it usually?

7. Please rate your like/dislike of the yoghurt with a cross on the scale below:

STRONG
DISLIKE

No OPINION

STRONG
LIKE

8. Would you continue to consume this yoghurt:
because of its taste? _____

if it was found to provide health benefits? _____

9. Are there any particular features of the yoghurt that you did or didn't like (eg, taste, texture, sweetness etc)? Please describe.

10. Please describe the fat content that you perceived the yoghurt to be (full fat, reduced fat, or no fat):

11. Any other comments about the yoghurt?

D. STUDY BREAD (only answer if we provided you with bread for the study)

1. Were you given bread for the 1st or 2nd 5-week period? _____

2. How many slices of this bread did you consume on average per day?

3. At which meal/snack time(s) did you consume the bread? How many slices at each time and how did you normally consume it (eg, toast, sandwich etc)?

5. Did you consume any other bread in addition to what we gave you?

6. What bread did you normally consume before the study?

7. In the other 5-week period where we did not supply bread, what bread and how many slices per day did you consume?

8. Please rate your like/dislike of the bread with a cross on the scale below:

STRONG
DISLIKE

No OPINION

STRONG
LIKE

9. Would you continue to consume this bread:

because of its taste? _____

if it was found to provide health benefits? _____

10. Are there any particular features of the bread that you did or didn't like (eg, texture, dryness, heaviness)? Please describe.

11. Did your bowel movements change while consuming this bread, and was this favourable?

12. Overall, did you feel any other health-related changes associated with consuming this bread?

E. GENERAL QUESTIONS AND COMMENTS

1. Did you enjoy participating in this study?

2. How easy did you find it to incorporate these foods into your diet?

3. Were the location and times convenient for you?

4. Did you think that the clinical area was adequate and professional?

5. Did you think that the study was conducted in a professional manner?

6. Would you consider taking part in another study, either similar or different at the university, and like to remain on our database?

**Are there any further comments that you would like to make?

Appendices 5.2 – 5.6 – landscape pages 208 – 212.

Appendix 5.2 Comparison between week 0 and week 9 plasma and urinary isoflavones. Mean \pm SEM.

Group Week			Plasma							Urine					
			(ng/mL)			ANOVA [†]				(mg)		ANOVA [†]			
			0 h	8 h	24 h	F	df	p	0 - 24 h	24 - 48 h	F	df	p		
daidzein	Pro-1	0	2.3 ± 2.3	160 ± 25	41.5 ± 13.5	0.814	1,9	0.391	12.9 ± 1.4	5.5 ± 1.9	1.083	1,9	0.325		
		9	4.8 ± 3.5	153 ± 22.1	30.1 ± 8.3				11.4 ± 1.9	4.4 ± 1.6					
	Pro-2	0	0	213 ± 37.9	30.6 ± 7.3	0.616	1,5	0.468	12.9 ± 1.5	3.8 ± 1.5	0.707	1,5	0.439		
		9	1.2 ± 1.2	184 ± 33.3	32.9 ± 4.9				10.5 ± 1.4	4.2 ± 2.3					
	Pre-1	0	1.5 ± 1.5	158 ± 28.7	36.3 ± 10.9	3.476	1,8	0.099	12.0 ± 1.3	2.4 ± 0.5	1.272	1,8	0.292		
		9	6.7 ± 6.0	192 ± 24.4	34.2 ± 9.3				12.0 ± 1.9	5.4 ± 1.6					
	Pre-2	0	1.7 ± 1.4	131 ± 20.0	45.0 ± 14.3	0.538	1,3	0.516	7.8 ± 1.4	2.1 ± 0.5	0.038	1,4	0.856		
		9	3.5 ± 1.8	159 ± 24.5	35.3 ± 4.9				8.1 ± 1.5	2.4 ± 1.0					
	genistein	Pro-1	0	3.2 ± 3.2	399 ± 50.5	133 ± 21.1	0.344	1,9	0.572	6.4 ± 0.9	2.2 ± 0.7	0.198	1,9	0.667	
			9	4.5 ± 2.6	375 ± 52.1	116 ± 22.7				5.2 ± 0.9	2.6 ± 1.4				
		Pro-2	0	0	471 ± 155	116 ± 31.6	0.014	1,5	0.910	6.8 ± 1.5	1.0 ± 0.3	0.162	1,5	0.704	
			9	4.1 ± 4.1	432 ± 123	134 ± 35.3				5.7 ± 1.1	2.8 ± 1.8				
Pre-1		0	0	391 ± 66.7	113 ± 13.3	3.756	1,8	0.089	6.8 ± 1.1	0.8 ± 0.2	4.733	1,8	0.061		
		9	0.73 ± 0.73	476 ± 71.6	174 ± 45.2				8.9 ± 2.0	3.6 ± 1.1					
Pre-2		0	4.8 ± 4.1	270 ± 50.3	73.8 ± 11.3	5.974	1,3	0.092	3.4 ± 0.6	0.6 ± 0.2	0.022	1,4	0.889		
		9	4.9 ± 2.6	382 ± 65.3	112 ± 16.3				4.0 ± 1.3	0.8 ± 0.2					

[†]Two-way ANOVA with repeated measures.

Appendix 5.3 Probiotic groups – plasma daidzein and genistein. Mean \pm SEM. Pro-1: n = 10; Pro-2: n = 6.

	Group	Week	Dietary Phase	Plasma Sample *			Treatment effect [†]			Between treatments [‡]		
				0 h	8 h	24 h	F	df	p	F	df	p
daidzein (ng/mL)	Pro-1	0	S + YC	2.4 ± 2.4	160 ± 24.5	41.5 ± 13.5	3.104	1,9	0.112	0.446	1,9	0.521
		5		52.2 ± 24.4	156 ± 25.5	38.1 ± 10.3						
		9	S + YP	4.8 ± 3.5	153 ± 22.1	30.1 ± 8.3	3.835	1,9	0.082			
		14		61.0 ± 21.0	133 ± 28.5	39.4 ± 8.6						
	Pro-2	0	S + YP	0	213 ± 37.9	30.6 ± 7.3	1.593	1,5	0.263	0.051	1,5	0.831
		5		74.7 ± 34.7	190 ± 25.2	26.3 ± 5.9						
		9	S + YC	1.2 ± 1.2 ^a	184 ± 33.3 ^a	32.9 ± 4.9 ^a	8.705	1,5	0.032			
		14		33.0 ± 20.7 ^b	254 ± 31.4 ^b	46.3 ± 28.8 ^b						
genistein (ng/mL)	Pro-1	0	S + YC	3.2 ± 3.2	399 ± 50.5	133 ± 21.1	2.386	1,9	0.157	1.925	1,9	0.199
		5		173 ± 58.0	394 ± 56.0	150 ± 39.9						
		9	S + YP	4.5 ± 2.6 ^a	376 ± 52.1	116 ± 22.7	6.646	1,9	0.030			
		14		155 ± 33.6 ^b	334 ± 54.3	142 ± 36.8						
	Pro-2	0	S + YP	0	471 ± 155	116 ± 31.6	13.133	1,5	0.017	0.000	1,5	0.984
		5		277 ± 106	512 ± 81.8	147 ± 30.3						
		9	S + YC	4.1 ± 4.1 ^a	433 ± 123 ^a	134 ± 35.3 ^a	8.289	1,5	0.035			
		14		132 ± 41.4 ^b	601 ± 109 ^b	217 ± 64.3 ^b						

* Values within treatment at the same time with different superscripts are significantly different ($p < 0.017$, post-hoc t-tests, Bonferroni correction).

[†] Treatment effect: two-way ANOVA with repeated measures.

[‡] Between-treatment effect: three-way ANOVA with repeated measures.

Appendix 5.4 Probiotic Groups – Effect of 5 weeks soy + control yoghurt (S + YC) and soy + probiotic yoghurt (S + YP) on urinary isoflavones. Mean ± SEM. Pro-1: n = 10; Pro-2: n = 6.

	Group	Week	Dietary Phase	Urine Sample			Treatment effect [†]			Between treatments [‡]		
				0 – 24 h	24 - 48 h	Total	F	df	p	F	df	p
daidzein (mg)	Pro-1	0	S + YC	12.9 ± 1.4	5.5 ± 1.9	18.4 ± 2.8	0.435	1,9	0.526	0.253	1,9	0.627
		5		14.0 ± 2.0	2.9 ± 0.5	16.9 ± 2.3						
		9	S + YP	11.4 ± 1.9	4.4 ± 1.6	15.8 ± 2.7	1.086	1,9	0.325			
		14		13.4 ± 1.4	4.8 ± 1.3	18.2 ± 2.3						
	Pro-2	0	S + YP	12.9 ± 1.5	3.8 ± 1.5	16.7 ± 3.0	0.514	1,5	0.505	0.241	1,5	0.644
		5		15.5 ± 3.6	4.9 ± 1.1	20.3 ± 4.4						
		9	S + YC	10.5 ± 1.4	4.2 ± 2.3	14.7 ± 1.9	1.105	1,5	0.341			
		14		12.0 ± 1.3	7.3 ± 3.4	19.3 ± 4.3						
genistein (mg)	Pro-1	0	S + YC	6.4 ± 0.9	2.2 ± 0.7	8.6 ± 1.3	0.039	1,9	0.848	0.002	1,9	0.963
		5		7.6 ± 1.4	1.2 ± 0.4	8.8 ± 1.7						
		9	S + YP	5.2 ± 0.9	2.6 ± 1.4	7.8 ± 1.8	0.846	1,9	0.382			
		14		7.0 ± 1.0	2.6 ± 0.8	9.6 ± 1.6						
	Pro-2	0	S + YP	6.8 ± 1.5	1.0 ± 0.3	7.7 ± 1.8	2.094	1,5	0.208	0.588	1,5	0.478
		5		8.0 ± 2.1	2.4 ± 1.1	10.5 ± 3.0						
		9	S + YC	5.7 ± 1.1	2.8 ± 1.8	8.5 ± 2.0	1.329	1,5	0.301			
		14		7.7 ± 1.3	6.2 ± 3.9	13.9 ± 5.1						

[†] Treatment effect: two-way ANOVA with repeated measures.

[‡] Between-treatment effect: three-way ANOVA with repeated measures.

Appendix 5.5 Prebiotic groups - plasma daidzein and genistein. Mean \pm SEM. Pre-1: n = 9; Pre-2: n = 5.

	Group	Week	Dietary Phase	Plasma Sample *			Treatment effect [†]			Between treatments [‡]		
				0 h	8 h	24 h	F	df	p	F	df	p
daidzein (ng/mL)	Pre-1	0	SC	1.5 ± 1.5	158 ± 28.7 ^a	36.3 ± 10.9	14.577	1,8	0.005	1.856	1,8	0.210
		5		55.1 ± 18.6	195 ± 28.9 ^b	34.4 ± 11.1						
		9	S + RS	6.7 ± 6.0	192 ± 24.4	34.2 ± 9.3	3.406	1,8	0.102			
		14		46.8 ± 15.4	204 ± 27.3	66.2 ± 20.8						
	Pre-2	0	S + RS	1.7 ± 1.4	131 ± 20.0	45.0 ± 14.3	2.660	1,3	0.201	1.413	1,3	0.320
		5		43.7 ± 9.9	142 ± 22.7	34.1 ± 9.2						
		9	SC	3.5 ± 1.8	159 ± 24.5	35.3 ± 4.9	3.384	1,3	0.163			
		14		21.6 ± 8.6	174 ± 31.6	48.4 ± 5.7						
genistein (ng/mL)	Pre-1	0	SC	0 ^a	391 ± 66.7 ^a	113 ± 13.3	24.138	1,8	0.001	1.282	1,8	0.290
		5		232 ± 71.5 ^b	494 ± 61.2 ^b	149 ± 24.4						
		9	S + RS	0.7 ± 0.7 ^a	476 ± 71.6 ^a	174 ± 45.2 ^a	8.127	1,8	0.021			
		14		173 ± 40.5 ^b	577 ± 82.8 ^b	252 ± 84.4 ^b						
	Pre-2	0	S + RS	4.8 ± 4.1 ^a	270 ± 50.3	73.8 ± 11.3	5.206	1,3	0.107	30.035	1,3	0.012
		5		117 ± 20.4 ^b	405 ± 100	133 ± 27.7						
		9	SC	4.9 ± 2.6 ^a	382 ± 65.3 ^a	112 ± 16.3 ^a	35.140	1,3	0.010			
		14		135 ± 20.6 ^b	450 ± 93.1 ^b	209 ± 49.4 ^b						

* Values within treatment at the same time with different superscripts are significantly different ($p < 0.017$, post-hoc t-tests, Bonferroni correction).

[†] Treatment effect: two-way ANOVA with repeated measures.

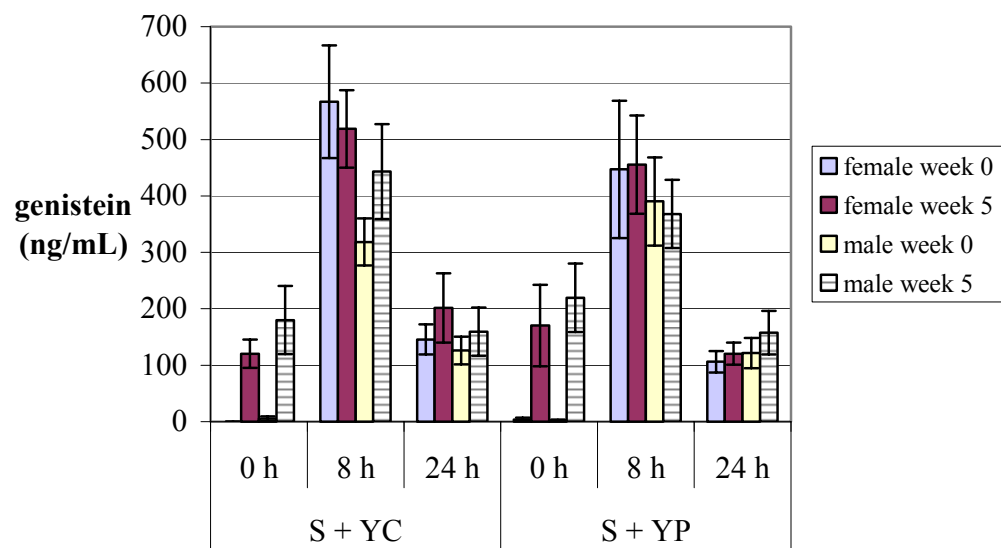
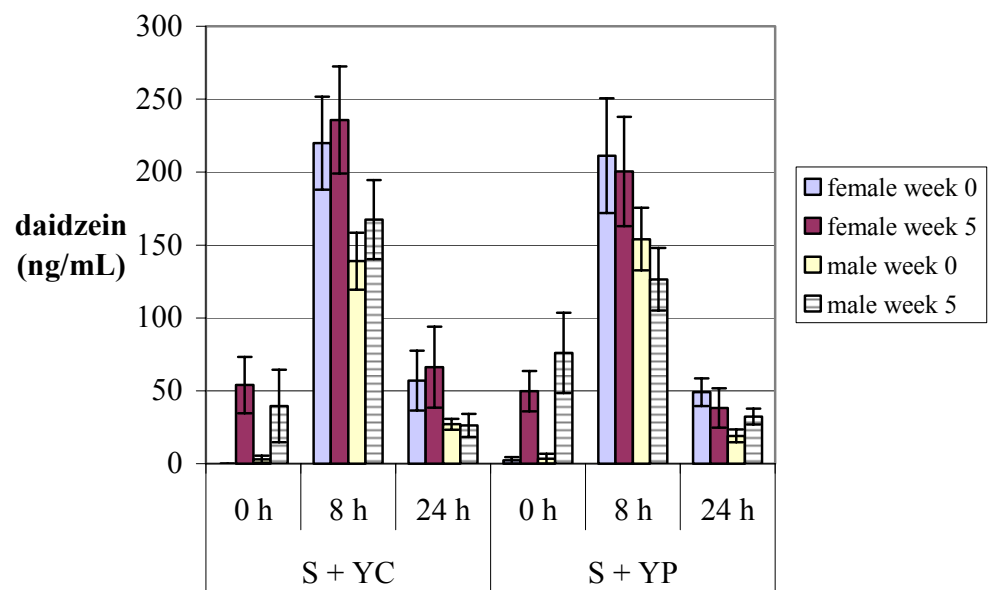
[‡] Between-treatment effect: three-way ANOVA with repeated measures.

Appendix 5.6 Individual Prebiotic Groups – Urinary daidzein and genistein after 5 weeks of soy control (SC) or soy + resistant starch (S + RS) intake. Mean \pm SEM. Pre-1: n = 9; Pre-2: n = 5.

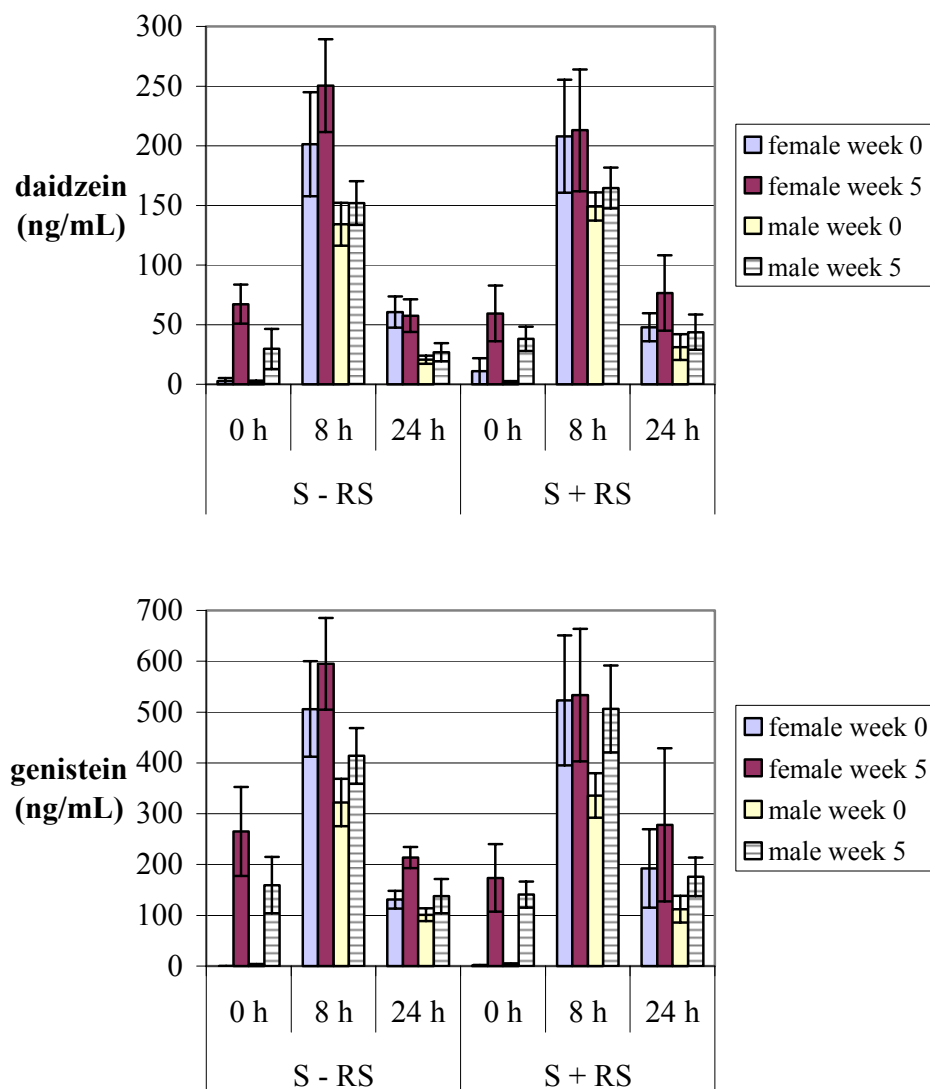
	Group	Week	Dietary Phase	Urine Sample			Treatment effect [†]			Between treatments [‡]		
				0 – 24 h	24 - 48 h	0 - 48 h	F	df	p	F	df	p
daidzein (mg)	Pre-1	0	SC	12.0 ± 1.3	2.4 ± 0.5	14.4 ± 1.5	0.889	1,8	0.373	0.639	1,8	0.447
		5		13.6 ± 3.3	5.0 ± 2.4	18.5 ± 5.3						
		9	S + RS	12.0 ± 1.9	5.4 ± 1.6	17.4 ± 3.0	0.302	1,8	0.597			
		14		13.4 ± 3.3	8.0 ± 3.2	21.4 ± 5.7						
	Pre-2	0	S + RS	7.8 ± 1.4	2.1 ± 0.5	9.5 ± 1.8	0.898	1,4	0.397	0.482	1,4	0.526
		5		11.0 ± 2.0	3.8 ± 1.1	14.4 ± 2.7						
		9	SC	8.1 ± 1.5	2.4 ± 1.0	10.1 ± 1.7	5.433	1,4	0.080			
		14		13.4 ± 3.1	4.8 ± 1.1	17.4 ± 2.5						
genistein (mg)	Pre-1	0	SC	6.8 ± 1.1	0.8 ± 0.2	7.7 ± 1.2	0.505	1,8	0.497	3.008	1,8	0.121
		5		7.3 ± 1.8	1.3 ± 0.6	8.6 ± 2.4						
		9	S + RS	8.9 ± 2.0	3.6 ± 1.1	12.5 ± 2.8	0.902	1,8	0.370			
		14		7.3 ± 0.8	2.8 ± 1.1	10.2 ± 1.7						
	Pre-2	0	S + RS	3.4 ± 0.6	0.6 ± 0.2	3.9 ± 0.7	2.038	1,4	0.227	0.618	1,4	0.476
		5		6.6 ± 1.6	2.9 ± 1.4	9.0 ± 2.8						
		9	SC	4.0 ± 1.3	0.8 ± 0.2	4.6 ± 1.4	9.187	1,4	0.039			
		14		6.9 ± 1.9	2.6 ± 0.8	9.1 ± 1.9						

[†] Treatment effect: two-way ANOVA with repeated measures.

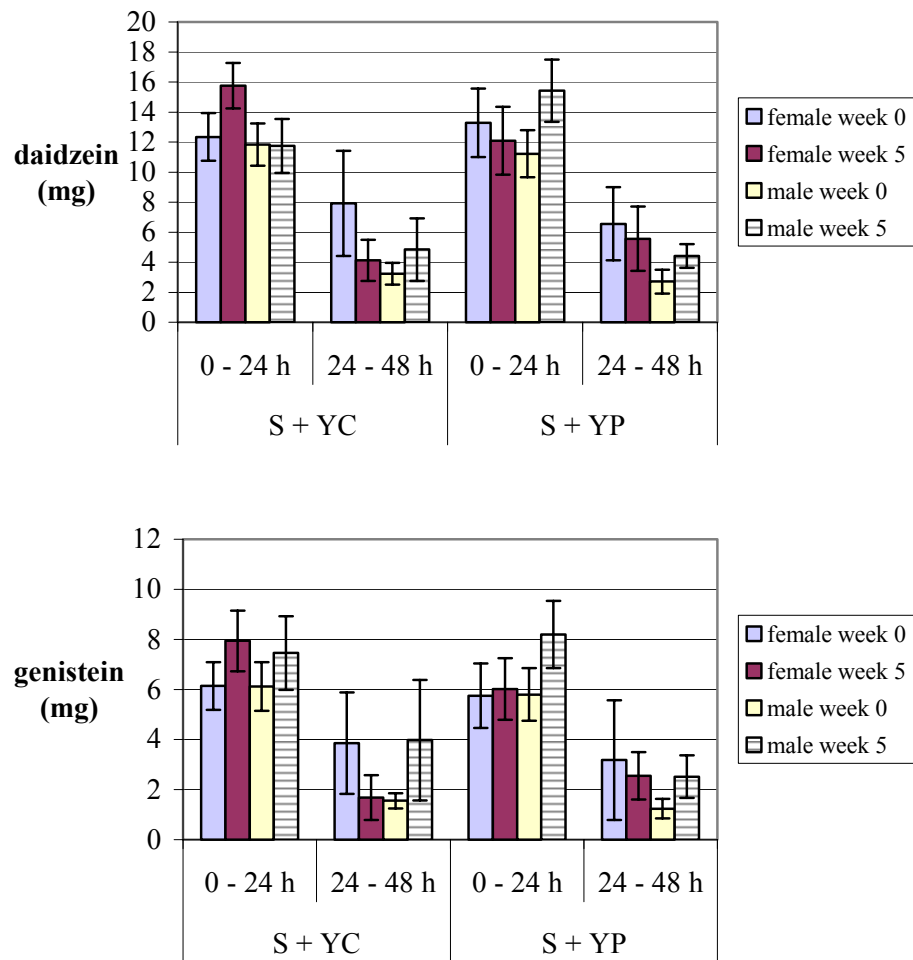
[‡] Between-treatment effect: three-way ANOVA with repeated measures.



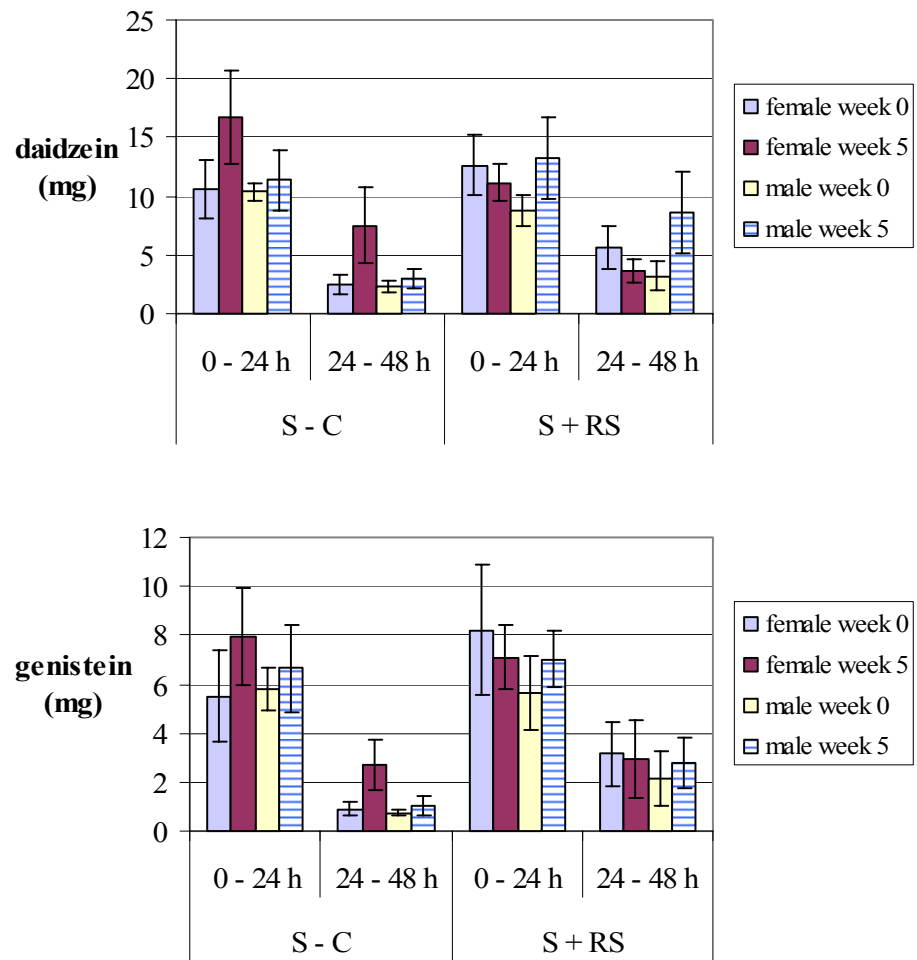
5.7A Gender differences in plasma daidzein and genistein for combined probiotic groups. Mean \pm SEM.



5.7B Gender differences in plasma daidzein and genistein for combined prebiotic groups. Mean \pm SEM.



5.7C Gender differences in urinary daidzein and genistein for combined probiotic groups. Mean \pm SEM.



5.7D Gender differences in urinary daidzein and genistein for combined prebiotic groups. Mean \pm SEM.

CHAPTER 6

COMBINATION OF SOY CEREAL AND A DHA-RICH OIL ON PLASMA AND URINARY ISOFLAVONES AND LIPIDS IN HYPERLIPIDEMIC SUBJECTS – A CONTROLLED, CROSSOVER STUDY.

Study Context

This dietary intervention was conducted by three PhD students in the Department of Biomedical Science and Smart Foods Centre at the University of Wollongong. We shared design for study design and implementation; Leisa Ridges conducted the clinic visits and analysed all cardiovascular and lipid data, Gina Martin conducted all dietary interviews and analyses and I processed all urine samples and analysed plasma and urine for isoflavone content. Although I did not conduct the lipid assays, I have used these raw data for my own analyses. Samples were also collected for antioxidant analysis of plasma and urine samples, but the development of these techniques and analyses was beyond the scope of this thesis.

6.1 INTRODUCTION

The n-3 and n-6 families of polyunsaturated fatty acids (PUFAs) are derived from alpha-linolenic acid (C18:3) and linoleic acid (C18:2), respectively. These are referred to as essential fatty acids as they cannot be made in the human body, but need to be obtained from dietary sources (Wijendran and Hayes, 2004). Eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are long chain n-3 PUFAs (Figure 6.1) found in fish and fish-oil (Holub, 2002). They are important structural components of membrane bound phospholipids in tissue throughout the body (Lee and Lip, 2003) and have cardiac, endothelial and lipid regulation effects, important in the prevention and treatment of coronary heart disease (Wijendran and Hayes, 2004).

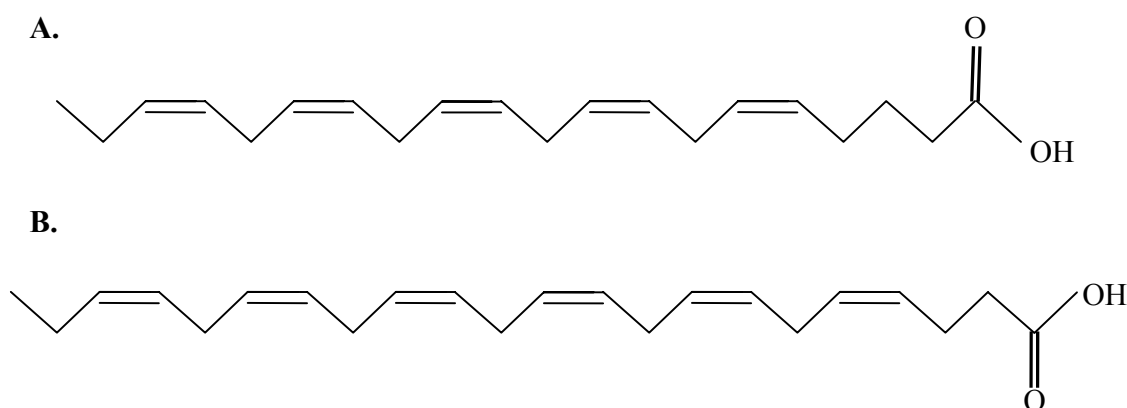


Figure 6.1 Chemical structure of n-3 polyunsaturated fatty acids: A. eicosapentaenoic acid (EPA, 20:5n-3) and B. docosahexaenoic acid (DHA, 22:6n-3).

Dyberg and co-authors (1975) reported the first epidemiological association between n-3 fatty acid intake and cardiovascular health. This concerned the Greenland Inuit population who, despite having a diet rich in fat, have very low mortality from coronary heart disease. This was attributed to the high n-3 fatty acid content of their diet, resulting from their habitual intake of fish, seal and whale. While Eskimos living in Greenland and consuming a traditional marine diet have a daily intake of 5 – 10 g of n-3 PUFAs,

mainly as EPA and DHA (Bonna *et al.*, 1992), n-3 PUFA intake in other European countries, the U.S. and Australia is generally less than 500 mg/day (Becker and Kyle, 1998; BNF, 1999; Kolanowski *et al.*, 2001; Sanders, 2000). The original association and cross-cultural epidemiological studies have clearly demonstrated an inverse relationship between the intake of n-3 PUFAs from fish and CVD death rates (Lee and Lip, 2003). In addition, fish oil supplementation between 1 and 2 g/day has been shown to reduce the risk of sudden cardiac death, the progression of cardiovascular disease and various cardiovascular disease risk factors as described in recent review papers (Din *et al.*, 2004; Harris *et al.*, 2003; Holub, 2002; Kris-Etherton *et al.*, 2002; Lee and Lip, 2003; Mori and Beilin, 2001; von Schacky, 2000; Wijendran and Hayes, 2004).

The most consistent physiological effect of dietary fish oil supplementation in relation to cardiovascular health is a dose-dependent reduction in fasting and postprandial triglyceride levels (Din *et al.*, 2004; Harris *et al.*, 2003). Significant reductions (25 – 30% in blood triglycerides have been observed within 2 - 3 weeks of EPA + DHA supplementation of 25 - 30% from a daily intake of between 2 and 4 g/day (Harris *et al.*, 2003; Holub, 2002; Kris-Etherton *et al.*, 2002; Stark *et al.*, 2000). However, this decrease in triglycerides is often accompanied by an increase in LDL cholesterol of between 5 and 10% (Angerer and Schacky, 2000; Harris *et al.*, 2003; Kris-Etherton *et al.*, 2002; Wijendran and Hayes, 2004). At higher n-3 PUFA doses, this increase may reflect an increase in LDL particle size which may be anti-atherogenic (Mori *et al.*, 2000; Mori and Beilin, 2001); however, the increase in LDL cholesterol is potentially a negative effect of n-3 supplementation. Some reports have indicated that DHA may be more important than EPA as the principal n-3 fatty acid in fish and fish oils responsible for the observed benefits on cardiovascular health (Mori and Beilin, 2001).

Consumption of n -3 PUFAs can increase the susceptibility of LDL and of membranes to oxidation (Harris *et al.*, 2003; Palozza *et al.*, 1996) and as membrane function is of vital importance to many cellular processes (Wiseman, 1999), protection of the membrane from free radical-mediated lipid peroxidation is desirable. It has been demonstrated that the pro-oxidant activities of n-3 PUFAs may be attenuated with concurrent antioxidant

supplementation (Trebbles *et al.*, 2003). While the main lipid effects of DHA supplementation are reductions in triglycerides, with increases in LDL, soy consumption may have complementary actions by reducing LDL, but with no effect on triglyceride levels. In addition, the antioxidant effect of soy isoflavones (Mitchell *et al.*, 1998) may also offer antioxidant protection against this negative pro-oxidative effect of n-3 fatty acid supplementation.

Thus, it was hypothesized that a dietary combination of soy and DHA supplementation may result in a reduction in both triglyceride and LDL levels with protection against the potential pro-oxidative effects of this n-3 PUFA through the antioxidant activity of the soy isoflavones. This study aimed to determine the effects of combined DHA supplementation and soy intake compared with soy alone on isoflavone bioavailability, lipids and other biomarkers of cardiovascular disease risk in hyperlipidemic men and postmenopausal women. In addition, isoflavone bioavailability after intake of a soy cereal higher in daidzein than genistein was determined.

6.2 METHODS

6.2.1 Subjects

Men and postmenopausal women older than 45 years were recruited through the local media. Inclusion criteria were total plasma cholesterol >5.0 mmol/L, plasma LDL cholesterol >3.5 mmol/L, triglycerides >1.6 mmol/L, mildly elevated blood pressure ($>140/90$ mm Hg) and no usage of lipid-lowering or anti-hypertensive medication in the past 6 months. Women needed to have been postmenopausal for at least 12 months prior to recruitment and not to have taken hormone replacement therapy (HRT) during the previous 6 months. Initially, subjects were selected by telephone interview and questionnaire. Potential participants then came in to the clinic for lipid and dietary screening after which only those whose lipid levels met the inclusion criteria and whose habitual diets did not include a high intake of soy-based foods or n-3 fatty acids were selected for participation. This study was approved by the Human Research Ethics Committee at the University of Wollongong (HE 00/222) and after screening, 47 subjects (29 males and 18 females) began the intervention.

6.2.2 Study design

This was a controlled, double-blind study of crossover design to compare the effects of two dietary constituents (DHA-rich oil and soy cereal) with corresponding controls (olive-oil and control cereal). Group allocation was based on screening lipids so that there would be similar baseline lipid levels between groups, but study investigators and participants were blinded as to which cereal and oil interventions each group followed. After a 3-week wash-in period during which subjects were instructed to avoid foods containing soy, subjects followed two 6-week dietary periods, not separated by a washout period (Figure 6.2). Two groups consumed olive oil for the entire 12 weeks, while the other two groups consumed DHA-rich oil for 12 weeks. One olive oil group and one DHA-rich oil group consumed the control cereal for the first 6 weeks while the other two

groups consumed soy cereal, then all groups crossed over to the alternate cereal for the following 6 weeks. Thus, there were two cohorts (olive oil or DHA oil) each made up of two groups, determined by the order of cereal intervention. Groups were identified by oil intervention cohort (OO = olive oil and DHA = DHA-rich oil) and by the order in which the two cereals were consumed (c-s = control cereal in the first 6 weeks followed by soy cereal for the second 6 weeks and s-c = soy cereal followed by control cereal).

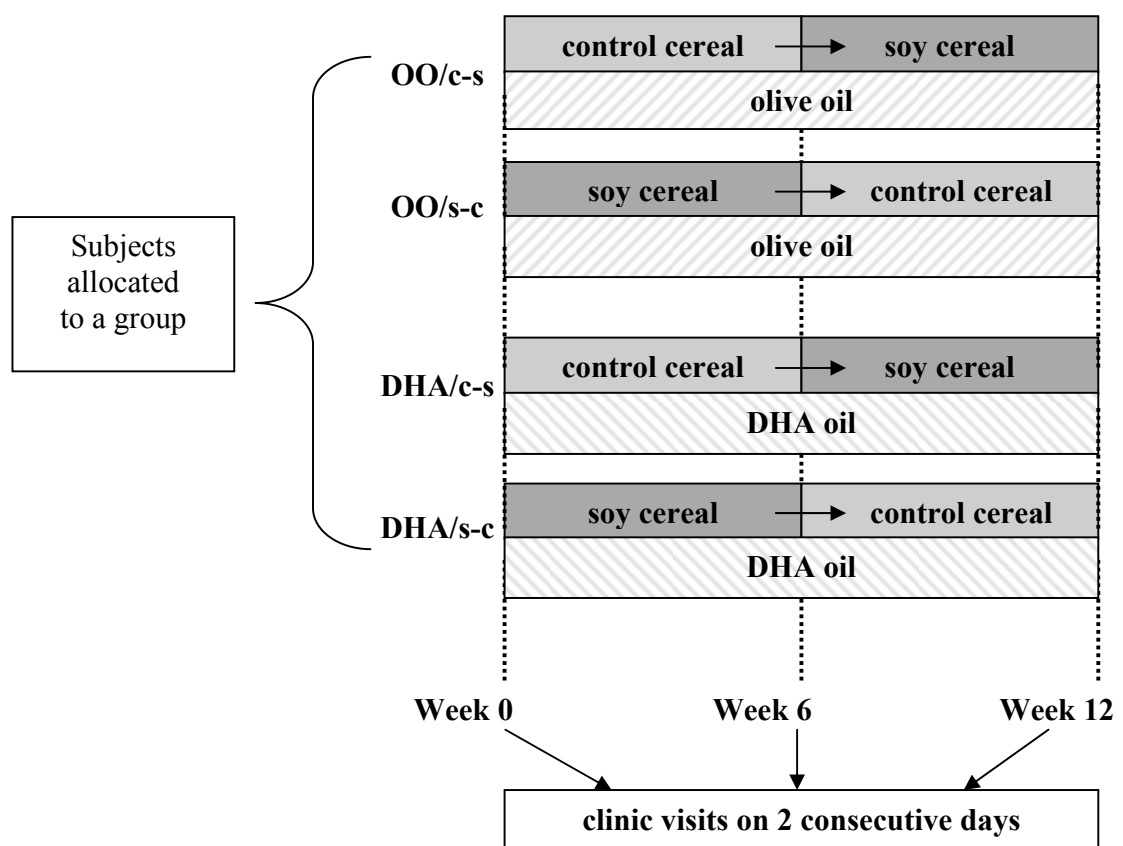


Figure 6.2 Study Design.

Prior to commencement of the study, all subjects attended an individual diet interview with a dietician to establish their habitual dietary intake and completed a 3-day food record. At week 0 (baseline) and weeks 6 and 12, subjects attended clinic visits on 2 consecutive mornings. On the first morning, height and weight were recorded, and clinic

blood pressure and arterial compliance were measured. A fasted venous blood sample was taken by a qualified nurse for determination of plasma levels of isoflavones, lipids, fatty acids and antioxidant status. For the 24 h between the first and second clinic visits, an ambulatory blood pressure monitor was worn (Model 90217, Spacelabs Medical Inc. Redmond WA, USA) and urine was collected into the 2.5 L vessels provided. On the second morning, a fasted blood sample was taken for a repeat lipid assay, the completed 24 h urinary sample was returned and subjects met with the dietician. In week 0, subjects were given advice on how to incorporate the cereal into their habitual diet (based on the initial diet interview) and were asked to maintain their usual use of oils and spreads and intake of fish, seafood and nuts. In weeks 6 and 12, a diet history was conducted for analysis of dietary nutrient composition during each dietary period. This thesis will only report on the isoflavone data and some lipid analysis as all the lipid and cardiovascular-related data and the dietary data are presented in two other PhD theses (L.Ridges, unpublished; G.Martin, 2004).

6.2.3 Study foods

All study foods were supplied by participating industry collaborators and subjects collected their oil and cereal at weeks 0 and 6 for consumption during the subsequent dietary period. For the oil supplementation, subjects were required to consume six capsules per day (1.2 g each) of either olive oil or DHA-Gold™ oil (both provided by Omega Tech, Boulder, Colorado, U.S.A.). The DHA content of the latter was 366 mg/g, which corresponded to a daily intake of 2.6 g DHA. The DHA-rich oil used was derived from a marine microalgae (*Schizochytrium* sp.) and is an approved novel food in Australia and New Zealand (FSANZ - Food Standards Australia New Zealand). The control and soy breakfast cereals were provided as pre-packaged 45 g serves (Specialty Cereals Pty. Ltd., Mt. Kuring-gai, NSW, Australia) for consumption of 1 serve per day. The soy cereal contained 3 g Soy Isolife™ per 45 g serve, which provided 90.3 mg isoflavones (46.4 mg daidzin + daidzein, 12.4 mg genistin + genistein, 31.5 mg glycitin + glycitein). The control cereal was prepared specifically for this study and had the same composition

as the soy cereal except without the Soy Isolife™. The cereal packages and containers of oil capsules were unmarked except for a numerical code.

6.2.4 Sample collection and analyses

Immediately after each blood sample was taken it was placed on ice until the end of each morning of clinic visits, when all samples were centrifuged at 3000 rpm at 4°C (Hettich, Universal 16R) for 10 min. Plasma was removed, and aliquots stored at –80°C prior to analysis. After the volume of each 24 h urinary sample was measured and recorded, aliquots were also stored at –80°C until analysis. Cholesterol content was determined for total plasma, triglycerides and isolated HDL using the standard commercially available kits (Roche Diagnostic) and an autoanalyser (Cobas Mira Plus). Plasma LDL cholesterol was calculated using the Friedewald calculation (Friedewald *et al.*, 1972). Isoflavones were extracted and quantified by HPLC-ECD as described in 2.3.2 and 2.3.3, respectively.

6.2.5 Statistical analyses

All data were analysed for normal distribution and presence of outliers (SPSS version 11.5, Chicago, Illinois). To determine whether there was an order effect of the dietary periods on any measures, one-way ANOVAs with repeated measures (ANOVA/RM) and between groups analyses were performed. Combined groups were analysed via one-way ANOVA with repeated measures and Bonferroni post-hoc analysis. When post-hoc analyses were not automatically reported, post-hoc Student's paired t-tests were used with a Bonferroni-correction. All Student's t-tests were 2-tailed. Correlations are reported as Pearson's correlation coefficient (R). Means are reported as \pm standard error of the mean (SEM).

6.3 RESULTS

6.3.1 Subject completion and group numbers

Although 47 subjects commenced the study, only 40 subjects completed the first 6-week dietary period (13 females and 27 males) and only 35 subjects (11 females and 24 males) completed the entire 12 weeks of the study. As each subject was acting as his or her own control, and paired comparisons or repeated measures were used in analyses, the data from subjects who only completed the first dietary period were excluded from the final set. All of the analyses, results and discussion are based on the 35 subjects who completed both dietary periods. The mean age at baseline was 52.6 ± 1.2 years and there were no differences between the four groups in either body mass or BMI during the study ($F = 0.173$, $p = 0.914$ and $F = 0.558$, $p = 0.646$, one-way ANOVA/RM and between groups analysis, Table 6.1). There was a significant (or a trend that approached being significant) effect of week on body and BMI for all groups (one-way ANOVA/RM, Table 6.1).

Table 6.1 Study Groups - subject distribution and anthropometric data. f = female, m = male.

Group	n	f / m		wk 0	wk 6	wk 12*	F	df	p [†]
OO/c-s	9	5 / 4	mass	85.7 ± 3.9	85.1 ± 3.8	85.7 ± 3.7	5.813	2,7	0.033
			BMI	31.9 ± 1.8	31.7 ± 1.7	31.9 ± 1.7	5.697	2,6	0.034
OO/s-c	8	1 / 7	mass	87.9 ± 5.4 ^a	88.9 ± 5.3	89.3 ± 5.4 ^b	8.179	2,7	0.019
			BMI	29.1 ± 1.4 ^a	29.5 ± 1.4	29.6 ± 1.4 ^b	7.540	2,7	0.023
DHA/c-s	9	3 / 6	mass	84.7 ± 3.1	84.6 ± 3.1 ^a	85.7 ± 3.2 ^b	10.054	2,7	0.009
			BMI	29.7 ± 1.5	29.7 ± 1.6 ^a	30.1 ± 1.6 ^b	9.216	2,6	0.011
DHA/s-c	9	2 / 7	mass	86.7 ± 4.0 ^a	87.4 ± 4.0	88.4 ± 4.3 ^b	4.198	2,7	0.063
			BMI	29.4 ± 1.3 ^a	29.6 ± 1.2	30.0 ± 1.4 ^b	3.989	2,7	0.070

*Values in the same row with different superscripts are significantly different, $p < 0.05$, Bonferroni post-hoc analysis.

[†]One-way ANOVA with repeated measures.

For all subjects, there was a significant influence of cereal intervention on body mass and BMI (Table 6.2); these were both increased after 6 weeks of soy cereal consumption compared with baseline ($p = 0.030$ and $p = 0.050$ respectively, Bonferroni post-hoc analysis). In male subjects, weight and BMI were both significantly increased after control and soy cereal interventions compared with baseline (weight: $p = 0.008$ and $p = 0.003$ respectively and BMI: $p = 0.010$ and $p = 0.006$ respectively, Bonferroni post-hoc analysis), but there were no effects for females. The BMI of females was significantly greater than that of males, but there was no gender difference in body mass (one-way ANOVA/RM and between groups analysis, Table 6.2).

Table 6.2 Anthropometric data for all subjects during control and soy cereal interventions. female, $n = 11$; male, $n = 24$.

	subjects	baseline	control	soy	ANOVA [†]			female vs. male [‡]
					F	df	p	
mass (kg)	all	86.2 ± 2.0 ^a	86.8 ± 2.0 ^a	86.9 ± 1.9 ^b	3.900	2,33	0.030	
	female	86.2 ± 3.9	86.0 ± 3.7	86.4 ± 3.7	1.312	2,9	0.316	F = 0.018 p = 0.893
	male	86.2 ± 2.3 ^a	87.1 ± 2.4 ^b	87.1 ± 2.3 ^{b*}	8.346	2,22	0.002	
BMI (kg/m ²)	all	30.1 ± 0.75 ^a	30.3 ± 0.74 ^a	30.3 ± 0.74 ^b	3.282	2,33	0.050	
	female	32.8 ± 1.6	32.7 ± 1.6	32.8 ± 1.6	1.225	2,9	0.338	F = 6.300 p = 0.017
	male	28.8 ± 0.68 ^a	29.1 ± 0.73 ^b	29.1 ± 0.70 ^{b*}	7.624	2,22	0.003	

[†]One-way ANOVA with repeated measures. Values in the same row with different superscripts are significantly different, $p < 0.05$, $*p < 0.01$, Bonferroni post-hoc analysis.

[‡]One-way ANOVA with repeated measures and between groups analysis.

6.3.2 Dietary intakes and compliance

After DHA intake, DHA as a percentage of total fatty acids in erythrocyte membranes had significantly increased from $4.63 \pm 0.21\%$ at baseline to $7.82 \pm 0.33\%$ and $9.32 \pm 0.29\%$ after 6 and 12 weeks, respectively. These were both significantly different to baseline ($p < 0.001$, Bonferroni post-hoc analysis, L.Ridges).

When all subjects were included in macronutrient analysis, there were significant overall effects of study duration on energy and carbohydrate intake, which both decreased over time. When groups were analysed separately, the only significant effects were on energy and CHO for the two olive oil groups and of MUFA for OO/c-s (Table 6.3).

When the data for the two olive oil groups were compared for baseline, control cereal and soy cereal interventions, OO/s-c had a significantly higher mean intake of all macronutrients excepting MUFA (energy: $F = 3.794$, $p = 0.020$, TF: $F = 4.925$, $p = 0.042$, SF: $F = 4.308$, $p = 0.056$, MUFA: $F = 4.871$, $p = 0.043$, PUFA: $F = 1.457$, $p = 0.246$, CHO: $F = 1.263$, $p = 0.279$, protein: $F = 1.090$, $p = 0.313$, one-way ANOVA/RM and between groups analysis). However, on a per kg body weight basis, there were no differences between groups in total energy, total fat, CHO or protein ($F = 1.546$, $p = 0.233$; $F = 2.565$, $p = 0.130$; $F = 0.479$, $p = 0.499$; $F = 0.383$, $p = 0.545$ respectively).

There were no significant differences between the two DHA groups for any of the macronutrient measures except for carbohydrate (energy: $F = 2.690$, $p = 0.120$, TF: $F = 0.591$, $p = 0.453$, SF: $F = 0.135$, $p = 0.718$, MUFA: $F = 0.898$, $p = 0.357$, PUFA: $F = 0.127$, $p = 0.726$, CHO: $F = 5.103$, $p = 0.038$, one-way ANOVA/RM and between groups analysis). Carbohydrate intake was greater for DHA/s-c at all time points. On a per kg body weight basis, there were no significant differences between the groups for total energy ($F = 1.264$, $p = 0.278$), total fat ($F = 0.105$, $p = 0.750$), CHO ($F = 3.616$, $p = 0.075$) or protein ($F = 3.443$, $p = 0.082$).

Table 6.3 Macronutrient data during study.

Group	Macronutrient				ANOVA [†]		
		Week 0	Week 6	Week 12*	F	df	p
All subjects	Energy (kJ)	10919 ± 388 ^a	10745 ± 377 ^b	10371 ± 383 ^b	6.596	2,33	0.004
	Total Fat (g)	90.8 ± 4.61	90.9 ± 3.99	88.5 ± 4.07	0.973	2,33	0.388
	SF (g)	30.6 ± 1.70	31.2 ± 1.67	29.9 ± 1.50	1.666	2,33	0.205
	MUFA (g)	34.1 ± 2.19	33.5 ± 1.75	32.9 ± 1.75	0.337	2,33	0.717
	PUFA (g)	17.7 ± 1.40	18.4 ± 1.28	18.1 ± 1.23	0.482	2,33	0.622
	CHO (g)	313 ± 12.6 ^a	308 ± 2.0 ^b	298 ± 12.7 ^{ab}	5.081	2,33	0.012
	Protein (g)	114 ± 4.5	112 ± 4.5	107 ± 3.9	6.092	2,33	0.006
OO/c-s	Energy (kJ)	9436 ± 774	9452 ± 776	8836 ± 742	6.810	2,7	0.023
	kJ/kg body weight	113 ± 10.8	113 ± 10.9	105 ± 10.3	4.595	2,7	0.053
	Total Fat (g)	73.2 ± 7.42	77.9 ± 7.18	71.6 ± 7.78	1.767	2,7	0.239
	SF (g)	25.3 ± 2.49	26.2 ± 3.19	23.3 ± 2.59	2.388	2,7	0.162
	MUFA (g)	26.2 ± 2.49 ^a	31.0 ± 2.96 ^b	29.1 ± 3.20 ^{ab}	6.184	2,7	0.028
	PUFA (g)	13.4 ± 12.2	13.2 ± 2.11	12.5 ± 2.09	0.979	2,7	0.422
	CHO (g)	282 ± 27.8	279 ± 24.5	265 ± 26.5	5.759	2,7	0.033
	Protein (g)	106 ± 8.9	104 ± 8.4	95.4 ± 6.3	4.574	2,33	0.054
OO/s-c	Energy (kJ)	11202 ± 729 ^{ab}	11472 ± 637 ^a	10833 ± 656 ^b	6.655	2,6	0.030
	kJ/kg body weight	131 ± 12.2	132 ± 10.9	125 ± 11.2	0.221	2,6	0.808
	Total Fat (g)	102 ± 11.9	98.4 ± 6.80	93.4 ± 6.83	0.260	2,6	0.260
	SF (g)	34.7 ± 4.68	32.5 ± 1.77	30.8 ± 1.87	0.462	2,6	0.462
	MUFA (g)	41.6 ± 6.56	41.2 ± 3.99	38.4 ± 3.86	0.193	2,6	0.193
	PUFA (g)	16.8 ± 1.73	16.1 ± 1.84	15.7 ± 1.51	0.581	2,6	0.581
	CHO (g)	302 ± 22.6 ^{ab}	335 ± 25.9 ^a	307 ± 24.8 ^b	0.029	2,6	0.029
	Protein (g)	115 ± 8.2	113 ± 9.7	113 ± 7.4	0.070	2,6	0.933
DHA/c-s	Energy (kJ)	10695 ± 651	10401 ± 612	10141 ± 621	1.802	2,7	0.234
	kJ/kg body weight	129 ± 10.3	125 ± 8.9	121 ± 9.0	1.603	2,7	0.267
	Total Fat (g)	89.2 ± 7.33	92.4 ± 6.31	90.9 ± 4.83	0.821	2,7	0.821
	SF (g)	31.2 ± 2.91	32.1 ± 2.67	31.6 ± 1.67	0.923	2,7	0.923
	MUFA (g)	31.1 ± 2.40	30.9 ± 2.37	30.8 ± 2.24	0.997	2,7	0.997
	PUFA (g)	18.8 ± 4.00	22.0 ± 3.38	21.5 ± 2.74	0.063	2,7	0.063
	CHO (g)	303 ± 19.3	284 ± 20.7	271 ± 22.9	0.157	2,7	0.157
	Protein (g)	105 ± 6.2	102 ± 6.4	99.8 ± 4.9	1.044	2,7	0.401
DHA/s-c	Energy (kJ)	12375 ± 702	11736 ± 809	11723 ± 775	3.398	2,7	0.093
	kJ/kg body weight	143 ± 7.1	133 ± 6.7	135 ± 7.9	3.132	2,7	0.107
	Total Fat (g)	101 ± 7.86	96.0 ± 9.45	98.5 ± 10.1	0.517	2,7	0.617
	SF (g)	31.7 ± 2.78	34.1 ± 4.65	33.9 ± 4.11	0.557	2,7	0.597
	MUFA (g)	38.4 ± 4.03	31.7 ± 3.88	34.1 ± 4.23	2.598	2,7	0.143
	PUFA (g)	21.7 ± 2.10	21.9 ± 1.18	22.5 ± 1.70	0.149	2,7	0.864
	CHO (g)	365 ± 24.0	339 ± 21.5	344 ± 21.8	1.897	2,7	0.220
	Protein (g)	131 ± 10.5	120 ± 9.5	128 ± 9.9	2.099	2,7	0.193

*Values with different superscripts are significantly different (p < 0.05, Bonferroni post-hoc analysis)

[†]One-way ANOVA with repeated measures.

6.3.3 Treatment of data

At baseline, there were no significant differences between the four study groups in plasma isoflavone levels (daidzein: $F = 0.960$, $p = 0.424$, genistein: $F = 0.276$, $p = 0.842$, equol: $F = 0.847$, $p = 0.479$, one-way ANOVA with between groups analysis) or for urinary daidzein or genistein excretion ($F = 2.001$, $p = 0.134$ and $F = 2.875$, $p = 0.077$, respectively, one-way ANOVA); equol was not detected in any subject's urine at baseline. There were also no differences between the four groups in plasma or urinary isoflavones after control cereal intervention (plasma daidzein: $F = 0.9602$, $p = 0.424$, genistein: $F = 0.842$, $p = 0.481$ and equol: $F = 1.496$, $p = 0.235$ and urinary daidzein: $F = 1.053$, $p = 0.383$, genistein: $F = 0.353$, $p = 0.787$ and equol, $F = 0.694$, $p = 0.563$, one-way ANOVA with between groups analysis). Thus, the description of plasma and urinary isoflavones at baseline and after control cereal intervention will include all subjects because there were no significant differences or trends between groups.

During the study (including at baseline and after control and soy cereal treatments), there were significant differences between the two olive oil groups in plasma daidzein and equol, but no other differences (Table 6.4) and no differences between the two groups of the DHA cohort (Table 6.5). Thus, the two groups of the olive oil cohort were originally analysed separately, while those of the DHA cohort were combined for analyses. As there was no crossover between the two oil interventions, the study design did not allow for intra-subject comparison of oil effects on soy isoflavone bioavailability or lipid levels.

6.3.4 Plasma and urinary isoflavones at baseline and after control cereal

At baseline, isoflavones were evident in the plasma of just over 50% of subjects (18 out of 35). Equol was the most prevalent plasma isoflavone at baseline, present in the plasma of 15 subjects (43%) at levels between 31.8 and 180 ng/mL. Ten subjects had plasma genistein (between 13.3 and 213 ng/mL) and 1 had plasma daidzein (95.0

ng/mL). One subject had all three plasma isoflavones, six others had both genistein and equol, three had just genistein and eight had just equol. All subjects excreted daidzein in their baseline urine samples (mean, 3.0 ± 0.43 mg; range, 0.3 - 10.2 mg) and 9 of these subjects also excreted genistein (between 0.1 and 1.2 mg), whilst no subjects excreted equol in their baseline urine.

After 6 weeks of control cereal intake, the mean plasma concentrations and urinary excretions of isoflavones were similar compared with baseline and there were 10 subjects (29%) whose plasma contained no isoflavones at either baseline or after control cereal. After control cereal, 19 subjects (54%) had low levels of plasma isoflavones and again, equol was the most prevalent isoflavone, present in the plasma of 18 subjects (51%), between 21.9 and 125 ng/mL. Seven subjects had plasma genistein (between 12.9 and 151 ng/mL) and 1 had plasma daidzein (35.9 ng/mL). One subject had all three plasma isoflavones, five others had both genistein and equol, one had just genistein and 12 had just equol. After the control cereal, 33 subjects excreted urinary daidzein (for all subjects, mean of 3.6 ± 0.49 mg; range, 0.0 – 13.0 mg), eight of these also excreted genistein (between 0.1 and 2.1 mg) but no equol, and two excreted equol (0.1 and 0.2 mg respectively) but no genistein. The remaining two subjects excreted no urinary isoflavones after control cereal intervention.

Table 6.4 Olive Oil Groups - plasma and urinary isoflavones. Mean \pm SEM (range), n = 15.

			Cereal Dietary Intervention [†]			Between groups [‡]		One-way ANOVA [†]		
group			baseline	control	soy	F	p	F	df	p
plasma (ng/mL)	daidzein	c-s	0	0	10.1 \pm 6.7 (0 – 48.6)	8.190	0.012	2.27	1,8	0.170
		s-c	0 ^a	0 ^a	73.8 \pm 22.5 (0 – 191) ^b			10.80	1,7	0.013
	genistein	c-s	17.1 \pm 7.2 (0 – 49.9)	6.3 \pm 6.3 (0 – 56.3)	34.2 \pm 11.7 (0 – 110)	0.740	0.403	2.19	2,7	0.183
		s-c	9.5 \pm 7.7 (0 – 62.4) ^a	4.1 \pm 4.1 (0 – 33.1) ^a	69.7 \pm 21.0 (0 – 155) ^b			5.38	2,6	0.046
	equol	c-s	30.6 \pm 15.6 (0 – 139)	30.1 \pm 10.7 (0 – 71.1)	30.1 \pm 17.9 (0 – 157)	4.435	0.052	0.324	2,7	0.733
		s-c	40.4 \pm 20.6 (0 – 134)	51.2 \pm 16.6 (0 – 125)	119 \pm 38.0 (0 – 285)			2.70	2,6	0.146
	urine (mg/24h)	c-s	1.8 \pm 0.46 (0.6 – 4.2) ^a	3.3 \pm 1.4 (0 – 13.0) ^a	8.1 \pm 2.2 (0.4 – 18.3) ^b	0.424	0.525	5.239	2,7	0.049
		s-c	3.2 \pm 1.1 (0.9 – 10.2) ^a	2.7 \pm 0.85 (0.7 – 6.7) ^a	9.9 \pm 1.2 (4.0 – 14.0) ^{b*}			12.23	2,6	0.008
	genistein	c-s	0.01 \pm 0.01 (0 – 0.10)	0.13 \pm 0.12 (0 – 1.1)	0.24 \pm 0.14 (0 – 1.2)	3.737	0.072	1.18	2,7	0.363
		s-c	0.34 \pm 0.18 (0 – 1.2)	0.20 \pm 0.14 (0 – 1.1)	0.96 \pm 0.42 (0 – 3.5)			3.22	2,6	0.112
	equol	c-s	0	0.01 \pm 0.01 (0 – 0.10)	1.1 \pm 1.1 (0 – 10.0)	0.038	0.848	1.00	2,7	0.415
		s-c	0	0	0.88 \pm 0.48 (0 – 3.6)			3.27	1,7	0.114

[†] One-way ANOVA with repeated measures. Values with different superscripts are significantly different (p<0.05, Bonferroni post-hoc analysis).

* Significantly different (p<0.01).

[‡] One-way ANOVA with repeated measures and between groups analysis.

Table 6.5 DHA groups - plasma and urinary isoflavones. Mean \pm SEM (range), n = 18.

			Cereal Dietary Intervention [†]			Between groups [‡]		One-way ANOVA [†]		
group			baseline	control	soy	F	p	F	df	p
plasma	daidzein	c-s	10.6 \pm 10.6	0	86.6 \pm 32.8	0.168	0.687			
		s-c	0	4.0 \pm 4.0	70.2 \pm 38.9					
		combined	5.3 \pm 5.3 (0 – 95.0) ^a	2.0 \pm 2.0 (0 – 35.9) ^a	78.4 \pm 24.8 (0 – 356) ^b			4.406	2,15	0.031
	genistein	c-s	23.7 \pm 23.7	16.7 \pm 16.7	77.6 \pm 24.4	0.271	0.610			
		s-c	8.9 \pm 4.5	28.6 \pm 15.2	48.4 \pm 18.8					
		combined	16.3 \pm 11.8 (0 – 213) ^a	22.7 \pm 11.0 (0 – 151) ^a	63.0 \pm 15.4 (0 – 205) ^b			3.645	2,15	0.051
	equol	c-s	11.6 \pm 8.4	16.4 \pm 8.8	11.0 \pm 7.4	1.998	0.177			
		s-c	45.1 \pm 18.6	28.5 \pm 9.8	129 \pm 93.7					
		combined	28.4 \pm 10.7 (0 – 180)	22.5 \pm 6.5 (0 – 75.9)	70.1 \pm 47.8 (0 – 867)			0.543	2,15	0.592
urine	daidzein	c-s	2.4 \pm 0.71	3.3 \pm 0.38	13.3 \pm 3.5	0.194	0.665			
		s-c	4.5 \pm 1.0	5.0 \pm 1.0	12.0 \pm 3.2					
		combined	3.4 \pm 0.65 (0.3 – 8.1) ^a	4.2 \pm 0.56 (1.4 – 10.5) ^a	12.6 \pm 2.3 (1.5 – 38.2) ^{b*}			6.943	2,15	0.007
	genistein	c-s	0.07 \pm 0.04	0.14 \pm 0.11	1.4 \pm 0.45	0.546	0.471			
		s-c	0.08 \pm 0.04	0.34 \pm 0.25	0.58 \pm 0.34					
		combined	0.07 \pm 0.03 (0 – 0.3) ^a	0.24 \pm 0.13 (0 – 2.1) ^a	0.97 \pm 0.29 (0 – 4.3) ^b			5.547	2,15	0.016
	equol	c-s	0	0.02 \pm 0.02	0	1.000	0.332			
		s-c	0	0	0					
		combined	0	0.01 \pm 0.01 (0 – 0.2)	0			1.000	1,16	0.332

[†] One-way ANOVA with repeated measures. Values with different superscripts are significantly different (p<0.05, Bonferroni post-hoc analysis).

* Significantly different (p<0.01).

[‡] One-way ANOVA with repeated measures and between groups analysis.

6.3.5 Isoflavones after 6 weeks of soy cereal

After the 6-week period in which soy cereal was consumed daily, only 20 subjects had measurable levels of plasma daidzein (57%), 25 had genistein (71%) and 17 had equol (49%). The only two subjects who had plasma daidzein after either baseline or control also had plasma daidzein after soy. Correspondingly, the plasma of 15 subjects did not contain daidzein at any of the three sample times during the study. Of the 10 subjects who had no plasma genistein after soy, nine of these also had no plasma genistein at any time. Eight subjects had neither daidzein nor genistein in their plasma at any sample during the study. There were only nine subjects who never had equol in their plasma at any time during the study, five of whom also had no plasma daidzein at any time. Of all the subjects who never had plasma genistein, their urinary excretion of genistein was less than 1 mg. After the soy cereal intervention, again all subjects excreted daidzein in their urine, 22 had urinary genistein and 4 had urinary equol.

For group OO/c-s, there were no overall significant effects of cereal treatment (one-way ANOVA/RM, Table 6.4) as levels after 6 weeks of soy intake (second dietary period) were not particularly elevated. However, an effect of cereal treatment approached significance for urinary daidzein as this was significantly greater after soy cereal compared with baseline. For OO/s-c, diet significantly affected plasma daidzein and genistein and urinary daidzein (one-way ANOVA/RM, Table 6.4), due to elevated levels after soy cereal (first dietary period) compared with both baseline and after control cereal.

For OO/c-s, there was a significant difference between the plasma concentrations of the three isoflavones ($F_{2,7} = 17.817$, $p = 0.002$, two way ANOVA/RM), due to significantly lower daidzein compared with both genistein and equol for all samples ($p = 0.009$ and $p = 0.006$ respectively, Bonferroni post-hoc analysis), but there were no differences for OO/s-c ($F_{2,6} = 2.934$, $p = 0.129$). There was a significant difference between the urinary concentration of the isoflavones for both olive oil groups (OO/c-s: $F_{2,7} = 7.553$, $p = 0.018$ and OO/s-c: $F_{2,6} = 20.090$, $p = 0.002$, two-way ANOVA/RM), due to urinary daidzein

being significantly greater than both genistein and equol (OO/c-s: $p = 0.013$ and $p = 0.029$ respectively and OO/s-c: $p = 0.001$ for both comparisons, Bonferroni post-hoc analysis).

For the DHA cohort, there was a significant effect of cereal treatment on plasma and urinary daidzein and genistein (one-way ANOVA/RM, Table 6.5), which was due to significant increases after the soy dietary period compared with both baseline and after the control cereal treatment. There were no differences between the plasma concentrations of daidzein, genistein and equol ($F_{2,16} = 0.240$, $p = 0.790$, two way ANOVA/RM) for the combined DHA groups, but there were differences between the urinary concentrations of these three isoflavones ($F_{2,16} = 26.375$, $p < 0.0001$, two way ANOVA/RM), as there was no equol urinary excretion and daidzein excretion was significantly greater than that of genistein ($p < 0.0001$ for daidzein compared with both genistein and equol and $p = 0.013$ between equol and genistein).

When the plasma and urinary isoflavone levels after soy intervention were compared for the two olive oil groups and the DHA cohort, there were no significant differences between them (one-way ANOVA, Table 6.6). There was a trend for similar plasma and urinary daidzein and genistein comparing the DHA cohort with OO/s-c but these levels were lower in OO/c-s. As the only significant differences between the two olive oil groups were in plasma daidzein and equol levels, which may have simply been due to the large variability that was evident in all groups, the two olive oil groups were combined for comparison between the two oil cohorts. Although there were no significant differences between the olive oil and DHA cohorts after soy cereal intervention in plasma or urinary isoflavones (Table 6.6), there was a trend for greater mean plasma and urinary daidzein and genistein with DHA supplementation. Mean plasma equol was similar between olive oil and DHA supplementation. After consumption of soy cereal, only 4 subjects excreted equol in their urine; these were all in the olive oil cohort.

Table 6.6 Plasma and urinary isoflavones after soy – comparison between two olive oil groups and combined DHA groups.

				Combined groups		One-way ANOVA [†]		t-test [‡]
		OO/c-s	OO/s-c	OO	DHA	F	p	p
Plasma (ng/mL)	daidzein	10.1 ± 6.7	73.8 ± 22.5	40.1 ± 13.4	78.4 ± 24.8	2.192	0.128	0.190
	genistein	34.2 ± 11.7	69.7 ± 21.0	50.9 ± 12.1	63.0 ± 15.4	0.988	0.384	0.544
	equol	38.1 ± 18.0	119 ± 38.0	76.0 ± 22.0	70.1 ± 47.8	0.556	0.579	0.912
Urine (mg/24h)	daidzein	8.6 ± 2.3	9.9 ± 1.2	9.2 ± 1.3	12.6 ± 2.3	0.827	0.446	0.216
	genistein	0.24 ± 0.14	0.96 ± 0.42	0.58 ± 0.23	0.97 ± 0.29	1.497	0.239	0.301
	equol	1.1 ± 1.1	0.88 ± 0.48	1.0 ± 0.61	0	1.408	0.259	0.102

[†]One-way ANOVA comparing 2 olive oil groups and combined DHA group.

[‡]Student's t-test comparing OO and DHA cohorts.

6.3.6 Metabolism of daidzein to equol

During the study, there were nine subjects who did not have measurable equol in plasma at any time during the study and 26 who had plasma equol at least once. Of these 26 subjects, there were 2 subjects who had plasma equol at baseline only, 3 after control cereal only, 3 at both baseline and control cereal and the remaining 18 subjects after soy cereal. The relatively common occurrence of equol in plasma or urine at times other than after soy intake hampered classification of subjects according to their equol-producing ability. However, for subjects who had plasma equol, but not after the soy dietary period, as their urinary daidzein excretion was increased after soy consumption, it appeared that the occurrence of equol in these subjects was not related to the ability to metabolise daidzein from soy. Thus, subjects were classified as “equol-positive” if they had equol in their plasma after soy intake or “equol-negative” if their plasma did not contain equol after soy intake. There were no significant differences between these two groups in plasma concentration or urinary excretion of either daidzein or genistein, although there was a trend for the plasma concentration of both daidzein and genistein to be greater in equol-positive subjects, (one-way ANOVA/RM and between groups analysis, Table 6.7). When dietary intakes of carbohydrate, starch and dietary fibre were

examined, there was a trend for equol-positive subjects to have a greater intake of starch than equol-negative subjects (Table 6.7).

Table 6.7 Plasma and urinary isoflavones for equol-positive and equol-negative subjects. n = 18 and n = 17, respectively.

						One-way ANOVA [†]		t-test [‡]
	equol		baseline	control	soy	F	p	p
plasma (ng/mL)	daidzein	+ ve	5.3 ± 5.3	2.0 ± 2.0	75.6 ± 23.7	1.640	0.209	0.267
		- ve	0	0	43.0 ± 15.9			
	genistein	+ ve	24.9 ± 12.0	22.2 ± 11.0	62.2 ± 12.9	1.965	0.170	0.602
		- ve	4.4 ± 4.4	5.7 ± 4.0	51.8 ± 15.1			
urine (mg/24 h)	daidzein	+ ve	3.7 ± 0.73	4.0 ± 0.76	10.1 ± 1.7	0.039	0.845	0.522
		- ve	2.2 ± 0.39	2.9 ± 0.33	11.9 ± 2.2			
	genistein	+ ve	0.12 ± 0.06	0.26 ± 0.14	0.72 ± 0.29	0.002	0.963	0.720
		- ve	0.12 ± 0.07	0.15 ± 0.08	0.85 ± 0.24			
Dietary intake	CHO	+ ve	321 ± 15.2	314 ± 13.6	318 ± 15.1	0.896	0.350	
		- ve	306 ± 20.1	293 ± 19.1	286 ± 20.6			
	starch	+ ve	176 ± 7.77	169 ± 8.62	168 ± 8.12	3.449	0.072	
		- ve	151 ± 10.5	145 ± 11.5	142 ± 13.0			
	DF	+ ve	33.3 ± 2.14	30.1 ± 1.66	29.7 ± 1.84	0.967	0.333	
		- ve	30.6 ± 1.73	28.1 ± 1.89	27.0 ± 2.05			

[†]One-way ANOVA comparing baseline, control and soy.

[‡]Student's t-test comparing equol-positive and equol-negative subjects after soy treatment.

6.3.7 Gender differences

There were no significant gender differences in plasma or urinary isoflavone levels, however there was a trend for males to have higher plasma genistein and equol but for women to have higher plasma daidzein after soy (Table 6.8). Correspondingly, the ratio of daidzein: genistein (D/G) in plasma was higher for females (2.0 ± 1.0 , $n = 7$) compared with males (1.1 ± 0.25 , $n = 18$), although this was not significant ($p = 0.242$, Student's t-test). The urinary D/G ratio also tended to be higher in females than males (20.8 ± 7.3 , $n = 6$ compared with 14.9 ± 2.9 , $n = 16$) due to females having similar levels of urinary daidzein but less urinary genistein than males, but again this was not significant ($p = 0.361$, Student's t-test). There were only a limited number of subjects whose plasma contained both daidzein and equol after 6 weeks of soy intake, for which the ratio of daidzein: equol was not statistically different between genders (males: 1.0 ± 0.44 , $n = 9$, females: 2.5 ± 2.0 , $n = 3$, $p = 0.282$, Student's paired t-test).

Table 6.8 Gender differences in plasma and urinary isoflavones. One-way ANOVA with repeated measures and between groups analysis).

			gender	baseline	control	soy	F	P
plasma	daidzein	f		0	0	80.7 ± 37.9	0.542	0.467
		m		4.0 ± 4.0	1.50 ± 1.50	50.2 ± 12.3		
	genistein	f		12.9 ± 6.1	5.1 ± 5.1	39.4 ± 12.4	1.311	0.260
		m		15.8 ± 9.1	18.4 ± 8.5	65.3 ± 12.9		
	equol	f		29.1 ± 13.9	17.1 ± 8.8	51.6 ± 27.7	0.510	0.480
		m		32.9 ± 10.0	34.4 ± 7.3	82.7 ± 36.6		
urine	daidzein	f		2.8 ± 0.72	3.9 ± 0.89	10.9 ± 2.0	0.001	0.977
		m		3.1 ± 0.55	3.3 ± 0.48	11.0 ± 1.8		
	genistein	f		0.01 ± 0.01	0.13 ± 0.10	0.43 ± 0.16	2.279	0.141
		m		0.17 ± 0.07	0.24 ± 0.11	0.95 ± 0.26		
	equol	f		0	0.02 ± 0.02	1.0 ± 0.90	1.519	0.226
		m		0	0	0.24 ± 0.17		

6.3.8 Lipids

At baseline, subjects were hyperlipidemic according to the National Heart Foundation of Australia Guidelines, 2004, with TCh > 6.0 mmol/L and LDL > 4.0 mmol/L (Table 6.8). There were no significant differences at baseline between the 4 groups in levels of total cholesterol (TCh), HDL cholesterol (HDL), LDL cholesterol (LDL) or triglycerides (TG) (Table 6.9).

Table 6.9 Baseline lipids for the whole study population (mmol/L). Mean \pm SEM.

	Group				between groups ANOVA		All subjects
	OO/c-s	OO/s-c	DHA/c-s	DHA/s-c	F	p	
TCh	6.7 \pm 0.82	6.1 \pm 0.40	6.1 \pm 0.36	5.9 \pm 0.30	0.363	0.780	6.2 \pm 0.25
HDL	1.0 \pm 0.14	1.0 \pm 0.11	1.2 \pm 0.09	1.1 \pm 0.07	0.662	0.581	1.1 \pm 0.05
LDL	4.3 \pm 0.69	4.1 \pm 0.26	4.0 \pm 0.45	2.2 \pm 0.30	0.598	0.621	4.1 \pm 0.22
TG	3.2 \pm 1.0	2.2 \pm 0.51	2.1 \pm 0.40	3.9 \pm 0.30	0.095	0.962	2.4 \pm 0.31

During the study, there were no significant differences between the two olive oil groups for any of the lipid measures (TCh: F = 0.105, p = 0.750, HDL: F = 0.019, p = 0.892, LDL: F = 0.357, p = 0.559, TG: F = 0.056, p = 0.816). Similarly, there were no significant differences between the two DHA groups (TCh: F = 0.918, p = 0.352, HDL: F = 0.331, p = 0.573, LDL: F = 0.534, p = 0.476, TG: F = 0.008, p = 0.931). Thus, the groups were combined as the OO and DHA cohorts for analysis.

When the two olive oil groups were combined, there were no significant differences between baseline, control cereal or soy cereal for TCh, HDL, LDL or TG. However, for the DHA cohort, there were significant effects of intervention on HDL, LDL and TG (Table 6.10). For subjects in the DHA cohort, HDL was significantly increased and TG was significantly reduced after both control cereal and soy cereal compared with baseline and LDL was significantly increased after the control cereal compared with baseline.

Table 6.10 Lipids during study for olive oil and DHA cohorts. Mean \pm SEM.

		Lipids (mmol/L) [†]			ANOVA		
		baseline	control	soy	F	df	p
Olive oil (n = 15)	TCh	5.93 \pm 0.22	6.19 \pm 0.25	5.97 \pm 0.27	1.490	2,13	0.261
	HDL	1.09 \pm 0.07	1.30 \pm 0.13	1.12 \pm 0.06	1.673	2,13	0.226
	LDL	3.94 \pm 0.15	3.78 \pm 0.26	3.83 \pm 0.20	0.240	2,13	0.790
	TG	1.97 \pm 0.28	2.44 \pm 0.51	2.22 \pm 0.45	1.554	2,13	0.248
DHA oil (n = 18)	TCh	6.04 \pm 0.23	6.34 \pm 0.20	6.17 \pm 0.19	2.206	2,16	0.143
	HDL	1.12 \pm 0.06 ^a	1.24 \pm 0.07 ^b	1.20 \pm 0.07 ^b	9.801	2,16	0.002
	LDL	3.95 \pm 0.26 ^a	4.38 \pm 0.21 ^b	4.21 \pm 0.20 ^a	3.784	2,16	0.045
	TG	2.12 \pm 0.24 ^a	1.57 \pm 0.18 ^b	1.66 \pm 0.20 ^b	5.942	2,16	0.012

[†] One-way ANOVA with repeated measures. Values with different superscripts are significantly different ($p < 0.05$, Bonferroni post-hoc analysis).

However, when DHA groups were originally analysed separately, there were some interesting temporal effects. There was a significant overall effect for DHA/c-s for TCh and LDL, which were both at their highest levels at week 6 after control cereal intervention (mean changes of $+0.51 \pm 0.21$ mmol/L and $+0.64 \pm 0.24$ mmol/L, respectively; Figure 6.3; Table 6.11). These lipids were increased during the first 6 weeks of DHA + control cereal but then decreased in the second 6 weeks with DHA + soy cereal intake. In contrast, when soy cereal was consumed during the first 6 weeks (group DHA/s-c), there was less of an increase in TCh and LDL (Figure 6.3; Table 6.11).

Although the changes in HDL and TG with the two consecutive 6-week dietary periods were similar for the two DHA groups, there were only significant differences for group DHA/s-c, perhaps due to the greater variance in group DHA/c-s (Table 6.11). When groups were combined, DHA supplementation resulted in significantly increased HDL after 6 and 12 weeks (by 8.8% and 9.4%, respectively) and significantly decreased TG after 6 and 12 weeks (by 19.8% and 18.4%, respectively). There was also a significant increase in LDL in the first 6 weeks of 14.5%.

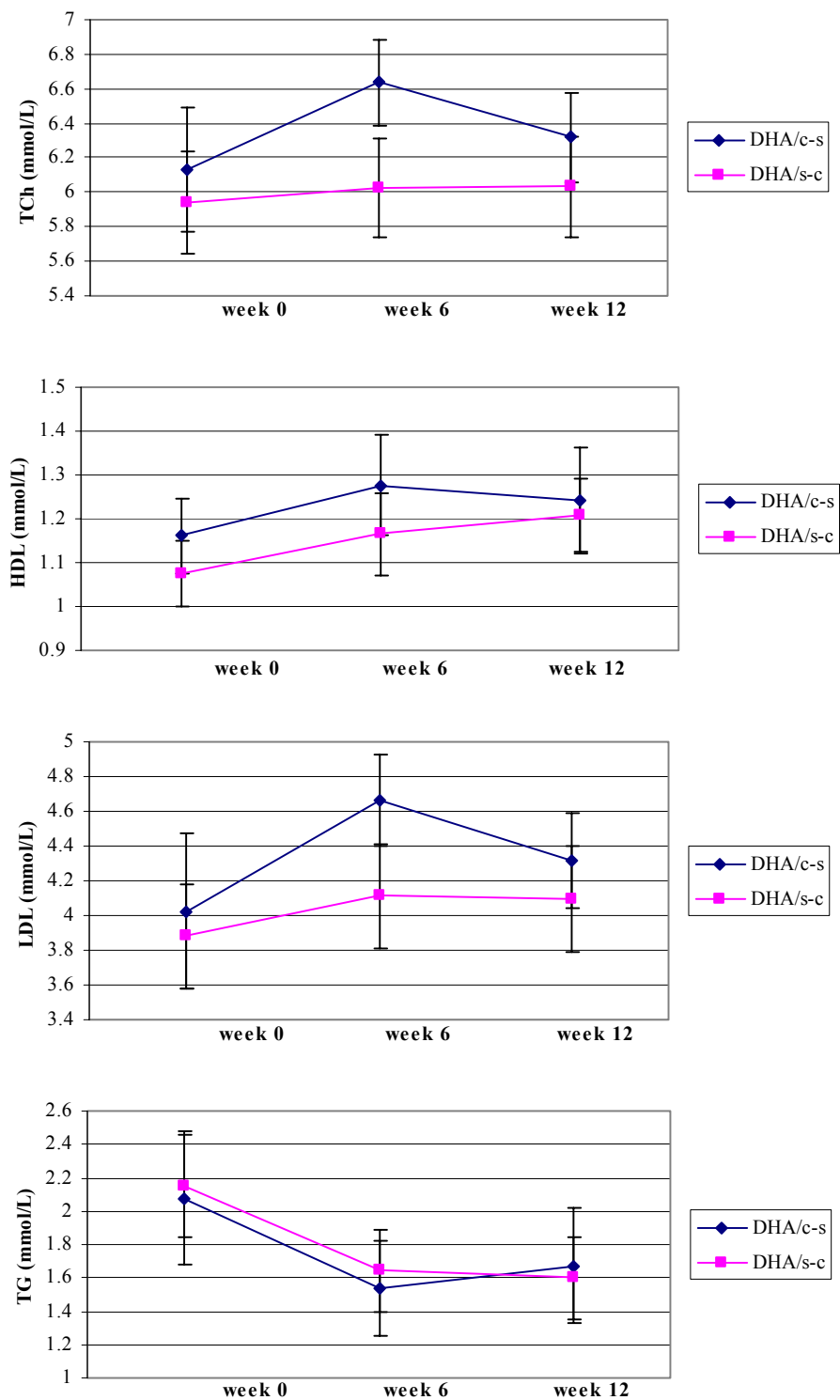


Figure 6.3 Lipid levels with DHA supplementation. DHA/c-s: control cereal weeks 0–6, soy cereal weeks 6 – 12. DHA/s-c: soy cereal weeks 0 – 6, control cereal weeks 6–12.

Table 6.11 Mean lipid levels during study for DHA cohort. Mean \pm SEM.

DHA group		Week [†]						Between groups
		0	6	12	F	df	p	
TCh	c-s	6.13 ± 0.36	6.64 ± 0.25	6.32 ± 0.26	5.007	2,7	0.045	F = 0.918 p = 0.352
	s-c	5.94 ± 0.30	6.03 ± 0.29	6.03 ± 0.29	0.338	2,7	0.724	
	combined	6.04 ± 0.23	6.33 ± 0.20	6.18 ± 0.19	2.796	2,16	0.091	
HDL	c-s	1.16 ± 0.09	1.28 ± 0.12	1.24 ± 0.12	2.977	2,7	0.116	F = 0.331 p = 0.573
	s-c	1.07 ± 0.07 ^a	1.17 ± 0.09 ^b	1.21 ± 0.08 ^b	26.790	2,7	0.001	
	combined	1.12 ± 0.06 ^a	1.22 ± 0.07 ^b	1.23 ± 0.07 ^b	7.365	2,16	0.005	
LDL	c-s	4.02 ± 0.45	4.66 ± 0.26	4.31 ± 0.27	6.440	2,7	0.026	F = 0.534 p = 0.476
	s-c	3.88 ± 0.30	4.11 ± 0.30	4.09 ± 0.30	1.834	2,7	0.229	
	combined	3.95 ± 0.26 ^a	4.39 ± 0.20 ^b	4.20 ± 0.20 ^{ab}	4.376	2,16	0.030	
TG	c-s	2.08 ± 0.40	1.54 ± 0.29	1.67 ± 0.34	1.561	2,7	0.275	F = 0.008 p = 0.931
	s-c	2.15 ± 0.30 ^a	1.64 ± 0.24 ^b	1.60 ± 0.24 ^b	13.407	2,7	0.004	
	combined	2.12 ± 0.24 ^a	1.59 ± 0.18 ^b	1.64 ± 0.20 ^b	4.838	2,16	0.023	

[†]One-way ANOVA with repeated measures. Values with different superscripts are significantly different (p < 0.05, Bonferroni post-hoc analysis).

Despite the significant lipid effects of DHA supplementation and the lack-there-of with OO supplementation, there were no significant differences between the two cohorts (TCh: F = 0.182, p = 0.673, HDL: F = 0.824, p = 0.370, LDL: F = 0.161, p = 0.690, TG: F = 3.164, p = 0.085, one-way ANOVA/RM and between groups analysis). The lipid changes for each dietary period are presented in Table 6.12. However, while small subject numbers and large variability may have masked statistical significance, the different effects on TG between the two oil cohorts may still be relevant, particularly given that the significant decrease in TG with DHA was evident even though this cohort had lower baseline triglycerides. While DHA-rich oil with control cereal significantly increased LDL and non-significantly increased TCh, the dietary combination of DHA-rich oil and soy cereal offered more beneficial lipid effects as this did not result in an increase in LDL and showed a trend toward a decrease in TCh (Table 6.10; Table 6.12).

Table 6.12 Change in lipids with 6 weeks of control cereal and soy cereal in olive oil and DHA cohorts. Mean \pm SEM.

		control cereal				soy cereal				soy vs. control
		Week 0	Week 6	mean difference	p	Week 0	Week 6	mean difference	p	p
Olive oil (n = 15)	TCh	5.99 \pm 0.25	6.19 \pm 0.25	0.20 \pm 0.17	0.252	6.04 \pm 0.24	5.97 \pm 0.27	-0.07 \pm 0.15	0.634	0.307
	HDL	1.11 \pm 0.06	1.30 \pm 0.13	0.19 \pm 0.15	0.224	1.13 \pm 0.07	1.12 \pm 0.07	-0.01 \pm 0.03	0.711	0.221
	LDL	3.83 \pm 0.17	3.78 \pm 0.26	-0.04 \pm 0.20	0.836	4.04 \pm 0.19	3.83 \pm 0.20	-0.20 \pm 0.12	0.109	0.503
	TG	2.32 \pm 0.44	2.44 \pm 0.51	0.12 \pm 0.16	0.478	1.91 \pm 0.29	2.22 \pm 0.45	0.31 \pm 0.31	0.328	0.614
DHA oil (n = 18)	TCh	6.08 \pm 0.22	6.34 \pm 0.20	0.25 \pm 0.15	0.114	6.29 \pm 0.21	6.17 \pm 0.19	-0.11 \pm 0.11	0.300	0.099
	HDL	1.16 \pm 0.06	1.40 \pm 0.07	0.08 \pm 0.03	0.025	1.18 \pm 0.07	1.20 \pm 0.07	0.03 \pm 0.02	0.210	0.297
	LDL	4.07 \pm 0.26	4.38 \pm 0.21	0.31 \pm 0.18	0.096	4.27 \pm 0.22	4.21 \pm 0.20	-0.06 \pm 0.12	0.650	0.164
	TG	1.86 \pm 0.23	1.57 \pm 0.18	-0.29 \pm 0.17	0.100	1.84 \pm 0.22	1.66 \pm 0.20	-0.19 \pm 0.13	0.156	0.680

6.3.9 Effects of equol production on lipids

There were no significant differences in baseline lipids between equol-positive and equol-negative subjects (TCh: $p = 0.106$, HDL: $p = 0.221$, LDL: $p = 0.081$, TG: $p = 0.553$, Student's t -tests), however there was a trend towards lower TCh and LDL in equol-positive subjects. When subjects were classified according to their equol-status after the soy dietary period, there were significant effects of diet on TCh, HDL and LDL for equol-negative subjects but no effects of diet for equol-positive subjects (one-way ANOVA/RM, Table 6.13). Overall equol-negative subjects had significantly higher LDL and almost significantly higher TCh during the study (one-way ANOVA/RM and between groups analysis, Table 6.13). For equol-negative subjects, TCh and LDL were significantly lower after soy compared with control but not with baseline, and HDL was significantly increased with both control and soy compared with baseline. There was also an interaction between group and treatment ($F_{2,32} = 6.085$, $p = 0.006$) as LDL was increased after soy compared with control in equol-positive subjects but decreased after soy compared with control in equol-negative subjects.

Table 6.13 Lipid levels for equol-positive and equol-negative subjects. Mean \pm SEM.

	equol	Lipid Levels (mmol/L)			ANOVA [†]			Between groups [‡]
		baseline	control	soy	F	df	p	
TCh	+ve	5.83 \pm 0.19	5.93 \pm 0.22	5.94 \pm 0.22	0.344	2,16	0.714	F = 3.874
	-ve	6.65 \pm 0.47 ^{ab}	6.83 \pm 0.29 ^a	6.48 \pm 0.31 ^b	3.897	2,15	0.043	$p = 0.057$
HDL	+ve	1.12 \pm 0.06	1.29 \pm 0.12	1.16 \pm 0.06	2.247	2,16	0.138	F = 0.983
	-ve	0.99 \pm 0.08 ^a	1.17 \pm 0.08 ^b	1.12 \pm 0.08 ^b	13.846	2,15	<0.0001	$p = 0.329$
LDL	+ve	3.68 \pm 0.15	3.70 \pm 0.20	3.91 \pm 0.16	2.166	2,16	0.147	F = 4.367
	-ve	4.46 \pm 0.42 ^{ab}	4.66 \pm 0.29 ^a	4.30 \pm 0.29 ^b	4.482	2,15	0.030	p = 0.044
TG	+ve	2.23 \pm 0.29	2.06 \pm 0.31	1.90 \pm 0.26	1.682	2,16	0.217	F = 0.336
	-ve	2.61 \pm 0.58	2.18 \pm 0.45	2.31 \pm 0.46	0.852	2,15	0.446	$p = 0.566$

[†] One-way ANOVA with repeated measures. Values in the same row with different superscripts are significantly different ($p < 0.05$, Bonferroni post-hoc analysis).

[‡] One-way ANOVA with repeated measures and between groups analysis.

6.3.10 Lipid correlations

There were no significant correlations between lipid levels at the beginning of, and their subsequent change with the soy cereal dietary period, although this approached significance for LDL (TCh: $R = -0.252$, $p = 0.158$, HDL: $R = -0.249$, $p = 0.163$, LDL: $R = -0.310$, $p = 0.079$, TG: $R = -0.003$, $p = 0.985$). However, the initial TCh level prior to the control cereal dietary period was significantly inversely correlated with changes in TCh and LDL ($R = -0.422$, $p = 0.014$ and $R = -0.395$, $p = 0.023$, respectively; Figure 6.4) and the initial LDL level was significantly inversely correlated with change in LDL ($R = -0.362$, $p = 0.038$). HDL and TG were not correlated with subsequent changes ($R = -0.298$, $p = 0.092$ and $R = -0.078$, $p = 0.666$, respectively).

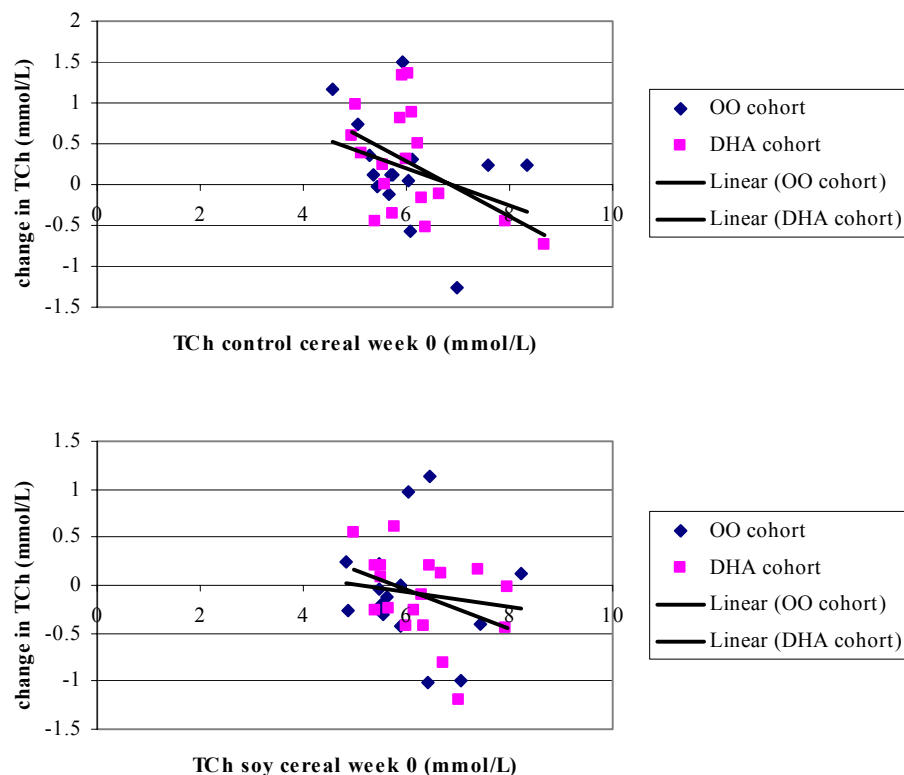


Figure 6.4 Correlation between TCh level before cereal intervention and the subsequent change during each 6-week intervention. $n = 33$.

In the DHA cohort, baseline TG level was correlated with the change after 12 weeks ($R = -0.550$, $p = 0.018$; Figure 6.5). However, when the outlying data of one subject who had a reduction in TG of 2.83 mmol/L was removed, this correlation only approached significance ($R = -0.455$, $p = 0.067$).

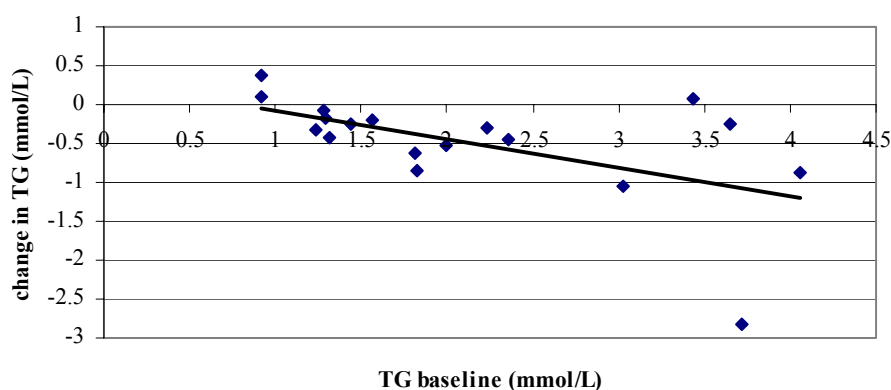


Figure 6.5 Correlation between TG and subsequent change with 12 weeks DHA supplementation. $n = 18$.

6.3.11 Gender differences in lipids

There were no significant gender differences in any lipid levels during the study, however there was a trend for females to have higher TCh, HDL and LDL but lower TG compared with males at all times during the study.

Table 6.14 Lipid levels per gender. f = female, $n = 11$; m = male, $n = 24$.

	gender	Lipid Levels (mmol/L)			Between groups [†]
		baseline	control	soy	
TCh	f	6.43 ± 0.71	6.58 ± 0.44	6.29 ± 0.47	F = 0.327
	m	6.13 ± 0.19	6.27 ± 0.19	6.16 ± 0.18	p = 0.571
HDL	f	1.10 ± 0.10	1.31 ± 0.10	1.24 ± 0.09	F = 0.965
	m	1.04 ± 0.06	1.20 ± 0.09	1.10 ± 0.06	p = 0.333
LDL	f	4.40 ± 0.57	4.53 ± 0.38	4.27 ± 0.40	F = 1.233
	m	3.91 ± 0.20	4.00 ± 0.21	4.02 ± 0.16	p = 0.275
TG	f	2.05 ± 0.54	1.61 ± 0.32	1.71 ± 0.37	F = 1.232
	m	3.58 ± 0.39	2.35 ± 0.35	2.28 ± 0.33	p = 0.275

[†]One-way ANOVA with repeated measures and between groups analysis.

6.3.12 Summary of results

Six weeks of daily intake of soy cereal significantly increased plasma and urinary isoflavone levels compared with baseline and after control cereal intake, with some trends of between-gender differences. In hyperlipidemic men and post-menopausal women, DHA-rich oil supplementation resulted in significantly increased HDL and decreased TG. While DHA-rich oil with control cereal significantly increased LDL and non-significantly increased TCh, the dietary combination of DHA-rich oil and soy cereal offered more beneficial lipid effects as this did not result in an increase in LDL and showed a trend towards a decrease in TCh. Overall there were no significant effects on lipids with olive oil but a trend toward an increase in TG. Olive oil with soy cereal caused a non-significant decrease in TCH and LDL, while TCh and HDL both increased with control cereal.

6.4 DISCUSSION

6.4.1 Plasma and urinary isoflavone levels at baseline and after control cereal

At baseline and after 6 weeks of control cereal intervention, the plasma of approximately 50% of subjects contained measurable levels of isoflavones and most subjects excreted one or more of daidzein, genistein or equol in their urine. This indicates that the diet of most subjects must have contained low to moderate levels of isoflavones or their precursors. The most prevalent plasma isoflavones at baseline and after control cereal intake were genistein and equol (Table 6.4; Table 6.5). The levels reported here of plasma daidzein and genistein after intake of habitual diets not supplemented with soy are similar to those reported for postmenopausal women in Italy (Albertazzi *et al.*, 1999) and in the U.S (Nettleton *et al.*, 2004), with mean daidzein 7.4 and 16.5 ng/mL, respectively and mean genistein 17.5 and 22 ng/mL, respectively, and for vegetarian Finnish women, 14 ng/mL daidzein and genistein (Adlercreutz *et al.*, 1993). The subjects in the present study had a rather high mean plasma level of equol compared with the vegetarian Finns (3.9 ng/mL; up to 10 ng/mL; Adlercreutz *et al.*, 1993), the Italian postmenopausal women (2.3 ng/mL and up to 51 ng/mL; Albertazzi *et al.*, 1999) and equol-positive subjects (3.4 ng/mL; Wiseman *et al.*, 2004).

Urinary isoflavone excretion at baseline and after control cereal was generally low, with mean 24 h excretion of 3.0 ± 0.43 mg and 3.6 ± 0.49 mg daidzein at baseline and after control cereal, respectively (up to 13 mg) and 0.1 ± 0.05 mg and 0.2 ± 0.08 mg genistein at baseline and after control cereal, respectively (up to 2.1 mg). Only one subject excreted equol, that being 100 µg after control cereal. Urinary isoflavone excretion in Anglo-Celtic subjects in Australia consuming their usual diets (Dalais *et al.*, 1998) and for subjects consuming various non-soy diets in the U.S. (basal, fruit and vegetable, legume, carotenoid or cruciferous has generally been reported at less than 1 mg/day daidzein, genistein or equol (Hutchins *et al.*, 1995a; Hutchins *et al.*, 1995b; Karr *et al.*, 1997; Kirkman *et al.*, 1995; Wiseman *et al.*, 2004). The higher urinary levels of

isoflavones in subjects at baseline in the current study may represent other sources of isoflavones and their precursors from the diet, including other legumes and sprouts (Liggins *et al.*, 2000).

6.4.2 Plasma and urinary daidzein and genistein levels after soy cereal intake

Despite the measurable levels of plasma and urinary isoflavones in the absence of dietary soy, daily consumption of soy cereal for 6 weeks significantly increased isoflavone absorption and excretion. The soy cereal provided a total of 90 mg isoflavones per day, of proportionally higher levels of daidzein and glycitein (46 and 31.5 mg, respectively) than genistein (12 mg). The total daidzein + genistein intake was 59 mg (D/G = 3.74) and when calculated per kg body weight, ranged from 0.41– 0.71 mg daidzein and 0.11 – 0.19 mg genistein/kg body weight (mean 0.54 mg daidzein and 0.14 mg genistein/kg body weight).

For the olive oil cohort, the mean plasma concentrations after 6 weeks of daily soy consumption were 40.1 ± 13.4 ng/mL daidzein, 50.9 ± 12.1 ng/mL genistein and 119 ± 38.0 ng/mL equol and for the DHA cohort were 78.4 ± 24.8 ng/mL daidzein, 63.0 ± 15.4 ng/mL genistein and 70.1 ± 47.8 ng/mL equol. After 6 weeks of soy cereal intake, there were no significant differences between these cohorts, and for all subjects, the mean plasma concentrations of daidzein and genistein were 59.8 ± 14.5 ng/mL and 57.1 ± 9.8 ng/mL respectively. As most subjects consumed the soy cereal with their morning meal, the plasma sample used for determination of isoflavone levels was taken approximately 24 h after their last soy cereal intake; however, there was a small number of subjects who consumed the cereal portion throughout the day. For all subjects after the soy dietary period, the average ratio of daidzein: genistein was 1.3 ± 0.34 (n = 25) in plasma and 16.5 ± 2.8 (n = 22) in urine.

These plasma isoflavone concentrations, approximately 24 h after the last soy intake, are comparable with other studies that have determined isoflavone levels 24 h after the last

intake of dietary soy supplementation. After consumption of soygerm, with a very similar genistein and daidzein content and dose to the current study (mean intake 36 mg daidzein and 10 mg genistein), plasma levels of 60 ng/mL daidzein and 47 ng/mL genistein were reported 24 h post-meal, also similar to the levels in the current study (Zhang *et al.*, 1999). After intake of texturised vegetable protein (TVP) containing proportionally less daidzein and more genistein than in the current study (26 mg and 30 mg respectively), subsequent plasma levels were also lower in daidzein, 51 ng/mL, and higher in genistein, 81 ng/mL, compared with those in the current study (Tew *et al.*, 1996). Plasma isoflavone levels are not only dependent on the amount contained in the food however, as plasma and urinary isoflavone levels are not always proportional to their levels consumed. For example, after intake of soy milk powder containing 46 mg daidzein and 36 mg genistein, plasma isoflavone levels were 20 ng/mL daidzein and 51 ng/mL genistein (Xu *et al.*, 1994) and after that containing 31 mg daidzein and 39 mg genistein, were 51 ng/mL and 120 ng/mL, respectively (Zhang *et al.*, 1999).

Although the plasma samples in the current study were not timed since the last soy intake, these fasted samples the day following soy intake would represent the lowest daily levels during the dietary period (approximately 24 h since last intake). Others have also used similar plasma levels to describe isoflavone bioavailability and the isoflavones are reported to continue to be absorbed for at least 12 h after intake. Watanabe, and co-authors (1998) and King and Bursill (1998) reported that total plasma concentrations remained above 50% of their maximum values for between 12 and 16 h after intake. In the current study after soy intake, the plasma levels of daidzein were greater than genistein (Table 6.4; Table 6.5). This is in contrast to the more common finding of the level of plasma genistein exceeding that of daidzein (King and Bursill, 1998), independent of the food, and even when the intakes of daidzein and genistein are equivalent (Setchell *et al.*, 2003b). The low proportion of genistein in the soy cereal used was most likely the contributing factor to its lower plasma levels. Most soy foods contain more genistein than daidzein (Franke *et al.*, 1995) and the disparity in plasma levels of the isoflavones probably simply reflects their relative doses in foods. Richelle and co-authors (2002) also reported a greater maximum concentration of daidzein than

genistein after intake of a soy drink containing either isoflavone aglycones or glucosides with a D/G ratio of greater than 4, similar to that of the current study.

The time since the last intake may also affect the ratio of daidzein: genistein in the plasma, as they have different pharmacokinetic properties; daidzein is typically cleared from the plasma faster than genistein (Setchell *et al.*, 2003a; Shelnutt *et al.*, 2002). This should result in proportionally less daidzein than genistein 24 h after the last soy intake. After three servings per day of isoflavones from soymilk powder in 8 females, Xu and co-authors (2000) found equivalent plasma concentrations of daidzein and genistein, although they did not specify the dietary proportions of the individual isoflavones. This is equivalent to 3 serves per day of the soy cereal that was provided in the current study, and the resulting plasma levels (650 and 360 ng/mL, after 6.5 and 24 h, respectively), were also much higher than levels reported here.

The mean urinary excretion for all subjects after 6 weeks soy cereal intake was 10.8 ± 1.4 and 0.78 ± 0.19 mg/24 h for daidzein and genistein, respectively. This clearly highlights the very small amount of genistein that was excreted, likely reflecting the low genistein content of the cereal. As subjects in the current study consumed their last serve of soy cereal at the beginning of the 24 h urine collection, it would be assumed that most of the absorbed isoflavones would be contained in this excretion sample. Lu and co-authors (1995) reported that more than 95% of daidzein and 83% of genistein were excreted within the first 24 h after soy ingestion; minimal amounts are present after 2 days (Setchell *et al.*, 2003b). Most other studies also report a higher urinary concentration of daidzein than genistein, although the large discrepancy between the 24 h excretions of daidzein and genistein in this study would again be related to the difference in the soy cereal.

The urinary isoflavone excretions reported here are similar to other studies using similar soy foods intakes. After single doses of soy foods, the 24 h urinary excretion of daidzein was 11 mg after a dose of 46 mg from soy milk powder (Xu *et al.*, 1994) and 9 mg after a dose of 36 mg from soy nuts (Setchell *et al.*, 2003b). Similarly, after a single dose of soy

milk powder providing 19 mg genistein (Xu *et al.*, 1994) and after intake of 9.8 mg genistein from soy nuts (Setchell *et al.*, 2003b), 24 h urinary genistein excretion was reported as 4 mg and 1 mg, respectively. Studies that have reported chronic intake of soy have generally used lower levels of dietary daidzein than in the current study, thereby limiting comparisons; however, after 9 days of soy intake providing between 11.5 and 20 mg genistein per day, urinary genistein excretion ranged between 0.46 and 0.70 mg/24 h (Hutchins *et al.*, 1995b; Karr *et al.*, 1997), similar to the current study. After consumption of a soy drink with a D/G ratio of greater than 4.0, Richelle *et al.*, (2002) reported similar urinary levels of daidzein (26 mg) and genistein (2 mg) to the current study. In random urinary samples taken from menopausal women during 12 weeks of daily supplementation with 60 g soy powder, Albertazzi and co-authors (1999) reported average excretion of 3.0 µg/mL daidzein and 2.8 µg/mL genistein, up to 11 µg/mL and 10 µg/mL, respectively. These are also comparable to the isoflavone excretion reported here, which equated to 6.4 ± 1.1 µg/mL daidzein and 0.83 ± 0.45 µg/mL genistein, up to 21 µg/mL and 11 µg/mL, respectively.

After 6 weeks of soy intake, both plasma and urinary daidzein and genistein were correlated ($R = 0.330$, $p = 0.053$ and $R = 0.438$, $p = 0.009$, respectively). In addition, plasma and urinary daidzein were correlated ($R = 0.371$, $p = 0.028$) and plasma and urinary genistein were correlated ($R = 0.597$, $p < 0.0001$). However, there were no correlations between daidzein and equol either in plasma ($R = 0.245$, $p = 0.156$) or in urine ($R = -0.087$, $p = 0.618$) nor between plasma and urinary equol ($R = 0.114$, $p = 0.516$). This suggests that despite large inter-individual variation, daidzein and genistein absorption and metabolism occurred similarly per individual, but that the metabolism of daidzein to equol is variable.

6.4.3 Metabolism of daidzein to equol

In this study, 51% of subjects produced equol after soy intake, which is a higher proportion than the 30 – 40% usually reported (Lampe *et al.*, 1998; Lampe *et al.*, 2001; Slavin *et al.*, 1998). This may have been due to the proportionally higher levels of daidzein than genistein in the soy cereal which would indicate a minimum amount of daidzein absorption may be needed for metabolism to equol. Tsangalis and colleagues (2002) also suggested that the formation of equol may be dependent on initial levels of daidzein. However, even at baseline, 43% of subjects had measurable equol in their plasma. The levels of equol reported in the current study covered a vast range (between 35 and 867 ng/mL). Fewer studies report plasma equol levels than urinary levels, and similar large variation in equol production determined by urinary equol excretion between individuals has been reported previously (Lampe *et al.*, 1998; Rowland, 1999). The urinary equol excretions of between 1 and 10 mg are comparable to those reported after 3 months of dietary supplementation with 60 g soy powder in menopausal women of 26 ng/mL in plasma and 1 µg/mL in urine (Albertazzi *et al.*, 1999). Similarly, after 5 days of soy intake, Axelson and Setchell (1980) reported urinary excretion of approximately 1 mg equol during the first 24 h after ingestion. However, in the latter study, maximum equol excretion, approximately 4 – 5 mg/day occurred after 3 days. In comparison to these levels and considering that the subjects in the current study had consumed soy for 6 weeks, it was expected that urinary equol excretion would have been higher.

Although not significant, there was a trend for females to have higher plasma daidzein and less equol than males. The increased levels of daidzein may be important if this means an increased bioavailability will allow daidzein to reach more tissues in which it may be further metabolized to equol. Alternatively, these combined results may suggest that there is enhanced conversion of daidzein to equol in men or more tissue retention of equol in women, the latter possibly being related to estrogen receptor distribution or greater percentage of fat (as women had a greater BMI than men). This result is similar to that reported in Chapter 5, but contrasts with the gender difference reported in Chapter

4, when men had higher plasma isoflavones. Thus, there appear to be differences between males and females in isoflavone bioavailability that may be related to the soy matrix or the additional effects of other dietary components.

6.4.4 Influence of oil on isoflavone bioavailability

When the two oil cohorts were compared, there was a trend for increased plasma and urinary levels of daidzein in the DHA groups and for the plasma D/G ratio to be higher with DHA compared with OO supplementation (1.9 ± 0.59 , $n = 13$ compared with 0.68 ± 0.17 , $n = 12$, $p = 0.066$, Student's t-test). However, there were no differences between the urinary D/G ratios between cohorts (15.0 ± 3.21 , $n = 13$ and 18.6 ± 5.3 , $n = 9$ respectively, $p = 0.548$, Student's t-test). Fish oil n-3 fatty acids can be incorporated into almost all lipoprotein classes (Clandinin *et al.*, 1997) and if the isoflavones were able to associate with DHA, they may have been incorporated into membranes, possibly altering their bioavailability. There was also a trend for a difference between cohorts in urinary equol excretion however, as after 6 weeks of soy cereal intervention; equol was only excreted by 4 subjects, none of which had followed DHA oil supplementation. This may suggest that DHA decreases the urinary excretion of equol, promoting its retention in the body.

6.4.5 Lipid effects

Subjects in this study were hyperlipidemic at baseline (TCh > 6.0 mmol/L and LDL > 4.0 mmol/L) based on the National Heart Foundation of Australia guidelines. Because these elevated lipid levels are major risk factors for CVD (Stark *et al.*, 2000), the subjects in this study may represent a subgroup of the population more receptive to dietary interventions with an aim of lipid lowering. In these subjects, there were significant reductions in TG and increases in HDL with DHA-oil intake that were unaffected by soy

intake. However, there were also effects on TCh and LDL that were enhanced with the dietary combination of DHA and soy.

As expected, 2.6 g/day of DHA supplementation significantly decreased TG, independent of the cereal intervention (Figure 6.3). There were no additional effects after the significant decrease in the first 6 weeks. The combination of DHA-rich oil and soy cereal resulted in a mean decrease in TG of 0.19 ± 0.13 mmol/L while DHA-rich oil and control cereal intake resulted in a mean decrease of 0.29 ± 0.17 mmol/L; thus there was no additional effect of soy cereal intake. At week 6 and week 12 of DHA supplementation, mean TG levels were significantly reduced by 19.8% and 18.4%, respectively. This is comparable with the change predicted by Holub (2002) of approximately 6 - 8% per gram of EPA + DHA consumed within 2 - 3 weeks and similar to other studies using DHA supplementation. Davidson and co-authors (1997) reported that 2.5 g/day DHA resulted in a significant decrease of 21% in TG levels in subjects with baseline TG of 2.95 mmol/L and Mori and colleagues (2000) reported that 4 g/day DHA significantly reduced TG by 18% in men with TG >1.8 mmol/L. Using an algae derived source of DHA (1.62 g/day), similar to that used in the current study, Conquer and Holub (1996) reported significant reductions in TG after 3 and 6 weeks of 22 and 16%, respectively in healthy vegetarians.

DHA supplementation also significantly increased HDL with both control and soy cereal interventions by 0.08 ± 0.03 mmol/L and 0.03 ± 0.02 mmol/L, respectively. Independent of cereal, significant increases were evident after 6 weeks (8.8%) and there were no additional significant reductions at 12 weeks (9.4%). Although an increase in HDL is not often reported with DHA supplementation, Davidson and colleagues (1997) reported a 6% increase in HDL with 2.5 g/day of DHA and Conquer and Holub (1996) reported a 16% increase with 1.62 g DHA/day. Non-significant increases in HDL were reported by Mori and co-authors (2000) and Theobald and co-authors (2004), while others have found no change in HDL with DHA-supplementation (Hamazaki *et al.*, 1996; Stark *et al.*, 2000), although mean HDL at baseline levels of these latter two studies were high (mean of at least 1.50 mmol/L).

While the effects of DHA supplementation on TG and HDL were not different between soy and control cereals, there did appear to be an additional benefit of soy cereal in combination with DHA. While LDL was increased by 11.3% with concurrent intake of DHA and control cereal, there was no elevation in LDL when DHA and soy cereal were consumed together (<1% change in LDL). In addition, with DHA supplementation, while there was a trend towards increased TCh (+4.9%) during the control cereal intervention, this was reversed (-1.4%) during the soy cereal intervention. Other studies have reported significant increases in LDL with DHA supplementation of up to 14% with intakes of DHA between 0.68 and 4 g/day (Davidson *et al.*, 1997; Mori *et al.*, 2000; Theobald *et al.*, 2004). A meta-analysis of fish oil supplementation as well as other studies have reported that TCh is unaffected by this supplementation (Conquer and Holub, 1996; Hamazaki *et al.*, 1996; Harris, 1997; Stark *et al.*, 2000; Theobald *et al.*, 2004). However, Harris (1997) reported a significant increase in TCh with DHA supplementation.

When the effects of DHA supplementation on TCh and LDL were carefully examined, there appeared to be a trend for soy cereal to attenuate the increases in these lipids that were observed with control cereal. During the first 6 weeks of DHA supplementation, TCh and LDL were increased by less when subjects were also consuming soy cereal rather than control cereal (Figure 6.3); thus, there may be an additional benefit of soy cereal in offsetting the increases in these lipids. Similarly, after increases in TCh and LDL resulting from DHA and control cereal consumption in the first 6 weeks, the crossover to soy cereal for the second 6 weeks ameliorated these increases (Figure 6.3). The proposed mechanisms for the effect of soy on LDL lipid lowering are via up-regulation of the LDL receptor or a decrease in LDL oxidation (Anderson, 2003; Mitchell *et al.*, 1998). As there was very little (<2 g) soy protein contained in the cereal used in the current study, the effects reported here from the soy cereal may be attributed to the isoflavones.

There were no significant lipid effects in the olive oil cohort independent of the concurrent cereal intervention, suggesting there were no lipid benefits of consumption of

the soy cereal alone. There was large inter-individual variation in triglyceride levels in this cohort with increased mean levels after 6 weeks in both cereal groups (Table 6.10). There were also trends towards increased TCh and HDL with the control cereal and decreased LDL with the soy cereal. Olive oil is generally the most common placebo used in studies with n-3 fatty acids, and from a meta-analysis of 36 studies, Harris (1997) reported negligible placebo treatment effects which more recent studies have also confirmed (Mori *et al.*, 2000).

Given that the 45 g daily serve of soy cereal contained less than 2 g soy protein, the lack of significant effect of the isoflavones alone from the soy cereal is not surprising. This daily intake of soy protein can be considered negligible, considering that the 38 clinical studies included in the meta-analysis of soy protein intake and serum lipids used between 17 and 124 g soy protein per day, with a mean of 47 g/day (Anderson *et al.*, 1995). Further, the FDA health claim (1999) indicates that soy protein may reduce blood cholesterol levels when consumed at a level of 25 g/day. Isoflavones given in the absence of soy protein have generally not resulted in significant lipid-beneficial effects, a result confirmed in a recent meta-analysis on the effects of isoflavones on serum lipids that found no significant changes in cholesterol with isoflavone tablets of up to 150 mg/day (Yeung and Yu, 2003).

However, a mean decrease in LDL of 0.20 ± 0.12 mmol/L (4.8%) after olive oil supplementation plus soy cereal intake from initial LDL levels of 4.04 ± 0.19 mmol/L is comparable to that reported by Crouse and colleagues (1999), who reported a decrease of 0.28 mmol/L (6%) with 25 g soy protein and 62 mg isoflavones. Although significant decreases in LDL of larger magnitudes have been attributed to the combined intake of soy protein and isoflavones (Cuevas *et al.*, 2003), other studies have failed to demonstrate a reduction in LDL with this same dietary intake (Kreijkamp-Kaspers *et al.*, 2004; Steinberg *et al.*, 2003). Although the reduction in LDL reported in the current study is not significant, it may still be of clinical interest and relevant in the context of other studies that have used dietary interventions including soy protein. The current

study suggests that soy protein was not necessary for some lipid effects, although it is not known whether additional soy protein may have enhanced the observed lipid effects.

When the olive oil and DHA cohorts were compared, although there were no significant differences between them for lipid effects, there were some notable trends. For both cohorts, there was a trend for a mean slight reduction in TCh with soy cereal (-0.07 mmol/L with olive oil and -0.11 mmol/L with DHA-oil) but a mean increase with control cereal (+0.20 mmol/L with olive oil and +0.25 mmol/L with DHA oil). This mean increase in TCH with olive oil plus control cereal may be accounted for by the concurrent increase in HDL, while DHA supplementation significantly increased HDL. The only significant effect on LDL was an increase with DHA supplementation and control cereal and the greatest mean decrease was during olive oil supplementation and soy intervention. There was a clear difference between oil cohorts in the effects on TG as this was increased with olive oil supplementation, but was decreased significantly with DHA-rich oil supplementation, both independent of cereal intervention. Thus, DHA had the traditional TG-lowering effect but there was no additional effect of isoflavones in this regard. The dose of DHA used was reasonable as effective doses of n-3 FA range from 3 to 5 g/d, which can only be obtained consistently by supplementation (Kris-Etherton *et al.*, 2002).

In the current study, baseline lipid levels were strongly correlated with lipid reductions after either control or soy cereal, suggesting this is the strongest predictor of a positive effect of dietary intervention. More elevated baseline TCh, LDL and TG were correlated with a greater reduction in these levels with both control and soy cereal, thus the effects were not dependent on soy intervention. Many soy intervention studies have reported greater lipid-lowering effects in individuals with more elevated baseline lipid levels (Crouse *et al.*, 1999), as was also reported in the meta-analysis (Anderson *et al.*, 1995). Similarly, previous studies with fish oil have shown the TG-lowering effects to be greatest in subjects with higher initial TG (Stark *et al.*, 2000).

In postmenopausal women, (Stark *et al.*, 2000) found no significant interaction between fish oil supplementation and hormone replacement therapy in lipid effects, however there was a trend for less reduction in triglycerides in the women using; this was not evident with soy supplementation in the current study. Using a dietary combination of soy and linseed foods, the latter high in α -linolenic acid (the plant precursor of DHA and EPA), Ridges and co-authors (2001) reported significant decreases in TCh and LDL after 3 weeks in 18 post-menopausal women with a mean baseline cholesterol of 6.44 mmol/L. These foods provided a daily intake of 45 mg isoflavones, 6 g α -linolenic acid and 32 mg lignans and resulted in mean reductions of 0.64 mmol/L TCh and 0.51 mmol/L LDL. However, this significant decrease was not maintained after a further 5 weeks. Although the change in TG was non-significant (from 2.06 to 1.92), there was a significant correlation between baseline triglyceride level and reduction after 3 weeks. Thus, although these significant lipid effects were noted with approximately half the daily intake of isoflavones provided in the current study, the linseed lignans or α -linolenic acid may have had resulted in synergistic effects, similar to the combination of soy and DHA in the current study. However, the conversion of α -linolenic acid to DHA and EPA is limited, and therefore the subsequent levels of DHA after ingestion of its precursor are not nearly as high as are achieved through dietary DHA supplementation. Also, there was not a significant reduction in TG levels in this study reported by Ridges and co-authors (2001), indicating insufficient conversion to DHA and/or EPA to produce significant TG lowering effects, as Harris (1997) noted that α -linolenic acid itself does not lower serum TG levels.

6.4.6 Conclusions and future directions

In this study population of hyperlipidemic men and postmenopausal women, there were beneficial effects of DHA-rich oil supplementation in relation to TG and HDL as these were significantly decreased and increased, respectively. The combination of DHA-rich oil and isoflavone-containing soy cereal did not further affect these changes; thus it

appears that DHA has a stronger effect on these lipids. However, there were benefits of the dietary combination of soy and DHA in relation to TCh and LDL, because while these lipids were both increased with DHA supplementation alone, significantly for LDL, these increases were attenuated with concurrent soy intake. Thus, there appear to be benefits of a dietary combination of soy and DHA, although there are no synergistic actions, a better balance in lipid effects resulted from this combination compared with either alone. Importantly, this effect occurred with only a small amount of soy protein, suggesting that the isoflavones in the soy cereal were the active components in this regard. This may be due to the antioxidant activity of the isoflavones, and would require further study using oxidation biomarkers for the elucidation of the mechanisms responsible.

CHAPTER 7

OVERALL DISCUSSION AND FINAL CONCLUSIONS

This thesis aimed to determine the effects of the soy matrix and other dietary components on soy isoflavone bioavailability and lipids, and to identify relationships between isoflavone bioavailability and lipid changes attributable to soy intake. This chapter will provide an overall discussion in which the major findings of the four studies of this thesis will be reviewed and compared in the context of the broader thesis aims. Final conclusions from the research conducted and recommendations for future research are also made.

For the purposes of this overview, the four studies reported in this thesis will be abbreviated:

S/RS = pilot study examining the effects of RS on isoflavone bioavailability
(Chapter 3)

S/D = crossover study comparing soy with dairy (Chapter 4)

S/PB = crossover study examining the effects of combination of soy with
a probiotic or prebiotic (Chapter 5)

S/O = crossover study of soy with DHA or olive oil (Chapter 6).

7.1 Influence of the Soy Matrix and its Isoflavone Content on Isoflavone Bioavailability

For the three studies in which chronic soy intake (5 or 6 weeks) was examined (S/D, S/PB and S/O studies), the proportions and concentrations of daidzein and genistein in the soy foods were evaluated for comparison with isoflavone bioavailability and lipid effects. Each study differed from the other two in the soy foods used, the proportions of

the isoflavones daidzein, genistein and glycitein, and the soy protein content, allowing for comparisons between these studies and the soy food composition (Table 7.1). In the S/D study, the source of soy was soy milk and/or soy yoghurt, of which most subjects consumed more milk than yoghurt; in the S/PB study, both soy milk and soy cereal were used; and in the S/O study, subjects consumed soy cereal only.

In the S/D and S/PB studies, the daidzein and genistein contained in the soy milk were present mostly as the β -glucosides, daidzin and genistin, with only 5.7 and 6.3%, respectively as the aglycones. However, 70% of daidzein and 75% genistein were present as aglycones in the soy yoghurt. The soy milk yoghurt used in the studies reported here had a daidzein and genistein content and D/G ratio within the range of those previously reported for soy milks (Barnes *et al.*, 1994; Coward *et al.*, 1993; Coward *et al.*, 1998; Eisen *et al.*, 2003; Franke *et al.*, 1998; Fukutake *et al.*, 1996). Coward and co-authors (1993) reported that soy milks typically contain less than 10% of aglycone isoflavones, as hot aqueous extraction of soybeans in soy milk production results almost entirely in the formation of β -glucosides (Barnes *et al.*, 1994; Coward *et al.*, 1998; King and Bursill, 1998). Although some researchers have reported differences between intake of aglycone compared with β -glucoside isoflavone supplements (Izumi *et al.*, 2000; Zubik and Meydani, 2003), Richelle and co-authors (2002) reported no differences in pharmacokinetics between a soy drink made from either a glycoside-rich or aglycone isoflavone extract.

Although the soy cereals used for the S/PB and S/O studies were manufactured by the collaborating industry partner specifically for these studies, the extracted soy product that was incorporated into the cereals differed, resulting in different isoflavone levels. For the S/PB study, a soybean extract (Nutragen 3-10) was used and contained 61% genistein, 31% daidzein and 5% glycitein, whereas for the S/O study, a soy germ-derived product (Soylife®) was used and contained 14% genistein, 51% daidzein and 35% glycitein. Soy germ (the hypocotyl) contains a greater proportion of daidzein and glycitein and much less genistein, which are correspondingly reflected in the isoflavone profile of products made from soy germ.

In the three studies (S/D, S/PB and S/O), the soy intake provided a similar total amount of isoflavones per day; however the individual intake of the specific isoflavones (daidzein, genistein and glycitein) varied (Table 7.1). The daily intake of daidzein was comparable between the three studies, however the genistein intake was lower in the S/O study and higher in the S/PB study. The glycitein content of the dietary soy was minimal in the S/PB study but constituted a third of the total isoflavone intake in the S/O study (Table 7.1). These dietary amounts of each isoflavone were reflected to a certain degree in the plasma and urinary isoflavone levels. When the S/D study and the control treatments in the S/PB and S/O studies were compared (a total of four control soy diets), plasma daidzein levels after soy intake were not significantly different between the three studies ($F = 0.726$, $p = 0.541$, one way ANOVA). Plasma genistein was more variable with a significant overall difference between the four soy diets ($F = 63787$, $p < 0.0001$, one way ANOVA), due to significant differences between the S/O study and both the S/D study and the control soy treatment (SC) of the S/PB study ($p < 0.0001$ and $p < 0.05$, respectively, Bonferroni post-hoc analysis). However, there was a trend for the plasma daidzein and genistein levels to be higher in the S/D study compared with the S/PB and S/O studies. Considering the daily intake of daidzein was similar between the studies and that the daily intake of genistein was less in the S/D study than the S/PB study, this may suggest enhanced plasma isoflavone absorption when provided in soy milk and/or yoghurt matrices.

Mean urinary excretion of daidzein was also similar between the three studies ($F = 1.620$, $p = 0.193$), however there was a trend for this to be lower in the S/O study. Urinary genistein was significantly lower in the S/O study ($F = 13.797$, $p < 0.0001$), compared with all other soy diets ($p < 0.001$, Bonferroni post-hoc analysis). There was also a trend for higher mean genistein excretion in the S/D study, further suggesting a particular increase in genistein bioavailability with soy milk intake. The significantly lower plasma and urinary genistein levels in the S/O study are directly related to the substantially lower genistein content of the soy cereal in the S/O study (Table 7.1), suggesting that the dietary intakes are the primary influence on subsequent isoflavone bioavailability.

Table 7.1 Isoflavone content of food and levels in plasma and urine after long-term intake in the studies in this thesis.

Study		S/D	S/PB				S/O	
Food	Soy Intake	soy milk	soy milk + soy cereal				soy cereal	
	Isoflavones	34 mg daidzein 48 mg genistein	38 mg daidzein 68 mg genistein 4.5 mg glycitein				46 mg daidzein 12 mg genistein 31.5 mg glycitein	
	D/G	0.71	0.56				3.8	
	Total isoflavones	82 mg	110.5 mg				89.5 mg	
	Soy protein	30 g	10 g				< 2 g	
	Dietary combination	soy*	control yoghurt*	probiotic yoghurt	control*	resistant starch	olive oil*	DHA oil
Plasma	daidzein	62.1 ± 8.4 (0 - 164)	45.0 ± 16.8 (0 - 260)	66.2 ± 17.8 (4.1 - 237)	43.1 ± 12.9 (0 - 162)	45.7 ± 10.4 (0 - 133)	40.1 ± 13.4 (0 - 191)	78.4 ± 24.8 (0 - 356)
	genistein	257 ± 33.8 (0 - 533)	158 ± 38.8 (0 - 514)	201 ± 45.5 (46.1 - 682)	197 ± 47.4 (58.3 - 598)	153 ± 27.7 (0 - 355)	50.9 ± 12.1 (0 - 155)	63.0 ± 15.4 (0 - 205)
	D/G	0.34 ± 0.05	0.35 ± 0.08	0.38 ± 0.08	0.20 ± 0.04	0.39 ± 0.10	0.68 ± 0.17	1.9 ± 0.59
	equol	n = 1/23 (32.1)	n = 3/10 (54.2 - 83.4)	n = 3/6 (33.1 - 202)	n = 6/9 (27.2 - 76.6)	n = 3/6 (31.0 - 122)	n = 10/15 (43 - 285)	n = 7/18 (35 - 867)
Urine	daidzein	12.4 ± 1.5 (0 - 25.9)	13.2 ± 1.3 (3.2 - 20.4)	14.2 ± 1.6 (4.0 - 27.8)	13.5 ± 2.3 (0 - 34.8)	12.4 ± 2.1 (4.2 - 39.0)	9.2 ± 1.3 (0.4 - 18.3)	12.6 ± 2.3 (1.5 - 38.2)
	genistein	9.5 ± 1.2 (0 - 21.3)	7.6 ± 1.0 (1.5 - 17.1)	7.4 ± 0.97 (0.81 - 16.9)	7.2 ± 1.3 (0 - 16.7)	7.0 ± 0.84 (2.3 - 13.4)	0.58 ± 0.23 (0 - 3.5)	0.97 ± 0.29 (0 - 4.3)
	D/G	1.43 ± 0.12	1.94 ± 0.17	2.20 ± 0.22	2.38 ± 0.44	2.05 ± 0.46	18.6 ± 5.3	15.0 ± 3.2
	equol	n = 1/23 (5.3)	n = 2/10 (0.4 - 4.4)	n = 2/6 (1.4 - 6.4)	n = 4/9 (0.41 - 3.8)	n = 1/6 (4.3)	n = 4/15 (1.2 - 10.0)	n = 0/18

*control soy treatments (for comparison in text).

The plasma D/G ratios were similar between the S/D and S/PB studies, however this ratio was higher in the soy food and lower in the urine in the S/D study, again suggesting increased genistein bioavailability from soy milk intake. Although the D/G ratio of soy cereal in the S/O study was 3.8, the plasma D/G ratio was 0.68 and the urinary D/G ratio 18.6, suggesting that a lower dietary intake of genistein reduced its urinary excretion more drastically than its levels in plasma. Further, while the soy intake in this study provided only 25% of the genistein content of the other two studies, the plasma genistein levels of genistein were approximately 50% and the urinary genistein excretion an order of magnitude lower. Thus, the levels of isoflavones in food are not always directly related to subsequent plasma and urinary isoflavone levels.

The mean 24 h urinary recoveries as a percent of intake, were similar for daidzein between the three studies: 36% in the S/D study, 30% in the S/PB study and 25% in the S/O study. For genistein, the mean percent 24 h urine recovery was similar for the S/PB and S/O studies, 6.5 and 7%, respectively, but was higher (20%) in the S/D study. This may be due to faster clearance of the isoflavones when provided in a liquid matrix as suggested by King and Bursill, (1998). In the S/PB study, of the total amount of daidzein excreted in the 48 h after soy intake, approximately 75% was excreted in the first 24 h, however for genistein, approximately 50% of the total 48 h amount was excreted in the first 24 h. Therefore, if due to faster plasma clearance and urinary excretion, proportionally more daidzein and genistein were excreted in the first 24 h in the S/D study, this would have a greater influence on genistein excretion and would contribute to increased 24 h recovery of genistein. Conversely, the lower recovery of genistein from the S/PB and S/O studies may suggest reduced bioavailability from the cereal matrix or increased metabolism to other unidentified compounds. The equivalent genistein recoveries from soy cereal that differed in genistein content by approximately 5-fold, implies that recovery was not affected by dose. The percent recoveries reported here are comparable to others in the literature (Table 7.2).

Table 7.2 Plasma and urinary isoflavone levels after soy intake.

Reference	Subjects	Source	Duration	Isoflavone Intake			Plasma			Urine (24 h)			
				D/G	Daily Dose (mg)		time	level (ng/mL)		mg excreted		recovery (%)	
					daid	gen			daid	gen	daid	gen	daid
(Zhang <i>et al.</i> , 2003)	6F	SM powder	single	1.18	21	24	3 h	1373	297			58	22
(Xu <i>et al.</i> , 1994)	12F	SM powder	single	1.3	25	19	6.5 h	201	200	4.8	0.97	20	5
							24 h	10.2	18.9				
			single	1.3	46	36	6.5 h	310	289	10.7	3.8	24	11
							24 h	20.3	51.3				
			single	1.3	71	56	6.5 h	569	581	14.2	5.4	21	10
							24 h	30.5	70.3				
(Zhang <i>et al.</i> , 1999)	7F	SM powder	single	0.88	31	39	6 h	254	459			45	25
	7M						24 h	51	108				
							6 h	330	486				
							24 h	51	135				
(Zhang <i>et al.</i> , 1999)	7F	Soy germ	single	3.8	36	10	6 h	407	135			40	26
	7M						24 h	56	43				
							6 h	295	127				
							24 h	61	51				
(Tew <i>et al.</i> , 1996)	7 F	Tofu	single	1.62	21	34	6.5 h	374	359	15	5.6		
		TVP	single	1.15	26	30	24 h	66	81				
							6.5 h	427	373	18	4.4		
							24 h	51	41				
(Setchell <i>et al.</i> , 2003)	10 F	Soy nuts	single	1.5	6.6	9.8	Tmax	109	158	2.5	1	63	25
					26	39	Tmax	420	597	9	4	44	16
(Nettleton <i>et al.</i> , 2004)	40F (PM)	Soy protein supplement	6 wks	0.59	15	25	12 h	58	20				
(Lampe <i>et al.</i> , 2001)	10F eql (+)	Soy protein powder	4 days	0.36	7.9	21.9				3.0	1.8		
	4 wks								3.3	2.8			
	9F eql (-)		4 days						5.0	2.2			
	4 wks								3.9	2.5			
(Wiseman <i>et al.</i> , 2004)	13 eql (+)	Soy foods	10 wks		104 mg total		>12 h	93.8	187	7.6	5.4		
	25 eql (-)							78.8	218	7.9	5.7		
(Hutchins <i>et al.</i> , 1995)	17M	Soybean pieces	9 days	0.44	8.6	19.6				1.0	0.46	5.7	1.3
		Tempeh	9 days	0.45	5.4	12.0				0.91	0.46	9.7	1.9
(Lampe <i>et al.</i> , 1998)	30F,30M	SP powder	4 days	0.36	8	22				3.1	1.7		
(Karr <i>et al.</i> , 1997)	7 eql (+)	Soy protein	9 days	0.56 1.77	6.5	11.5				1.6	0.70		
					13	23				2.4	1.4		
	7 eql (-)				6.5	11.5				1.8	0.97		
					13	23				3.5	2.0		
(Lu <i>et al.</i> , 1996)	6F	Soy milk	4 wks	0.87	99	114				39 - 90	4 - 50	66	24
(Lu <i>et al.</i> , 1995)	6M	Soy milk	4 wks	0.87	99	114				41 - 134	6 - 55	46	15

SM = soy milk, SP = soy protein. eql(+) = equol-positive subjects. eql(-) = equol-negative subjects. PM = postmenopausal.

If the enzymes required for isoflavone absorption and metabolism are saturable, then not only will the absolute dietary intakes be important, but so will the proportions of the different isoflavones in the soy matrix. This is particularly true for the conversion to secondary metabolites such as equol, which relies entirely on gut microfloral enzymes rather than on liver metabolism. There may be competition between isoflavones for metabolic pathways, an which has been suggested for the production of equol and ODMA from daidzein (Hutchins *et al.*, 1995; Kelly *et al.*, 1993). This is important as daidzein, genistein and glycitein, as well as their metabolites differ in their bioactivity. While genistein is generally considered more bioactive than daidzein (Sathyamoorthy and Wang, 1997), the physiological importance of equol may exceed that of both daidzein and genistein as discussed in Chapter 1. There is relatively less information about the bioactivity of glycitein and its metabolites, however Song and co-authors (1999) reported glycitein had equivalent affinity for the estrogen receptor as daidzein.

7.2 Effect of Other Dietary Components on Soy Isoflavone

Bioavailability

In the first study of this thesis, S/RS, acute resistant starch (RS) intake reduced plasma absorption and urinary excretion of daidzein and genistein, however chronic RS intake increased total urinary excretion of daidzein and its metabolites equol and ODMA. Therefore, apart from the soy matrix itself, other dietary constituents were also shown to affect isoflavone bioavailability. However, when the effect of chronic RS intake was further examined in the S/PB study, there were no significant effects on isoflavone bioavailability. There was the same trend for increased daidzein excretion in some people, however there was no increase in equol production as was observed in the S/RS study.

A trend for higher mean plasma isoflavones 24 h following soy intake after RS intake in the S/PB study again implied that RS could enhance or delay isoflavone absorption, however this was not evident after soy and RS had been consumed in the same day.

When the results of the S/RS and the S/PB studies are taken together, there appears to be an immediate effect of RS to impede isoflavone absorption when consumed in the same day but to increase isoflavone bioavailability when consumed the day prior to soy intake. This finding requires further investigation but does suggest a prebiotic action of RS in relation to isoflavone bioavailability. In addition, the trend for probiotic intake to increase circulating plasma isoflavones (daidzein, genistein and equol) in the S/PB study also implies a potential for probiotic bacteria. Probiotic intake affected circulating plasma isoflavone levels more than those 24 h after soy intake, while the converse was true for prebiotic intake. This indicates differences in gut microflora effects, as those from probiotic intake are short-lived compared with those resulting from prebiotic intake. Hence, there appears to be potential for probiotic or prebiotic intake to increase soy isoflavone bioavailability, although further investigation is required to determine optimal regimes of consumption.

7.3 Metabolism to Equol

There was wide variation in the occurrence of equol in the three long-term studies, although there was greater prevalence of equol in plasma than urine in all three. The ranges of plasma and urinary equol levels were also similar between studies. In the S/D study, only one subject had equol in their plasma or urine after soy intake. This may suggest that, if indeed isoflavone pharmacokinetics are faster after intake in a liquid matrix, there was insufficient opportunity for conversion of daidzein to equol from this food matrix. Zubik and Meydani (2003) also suggested longer transit time increases equol production. Conversely, whole soybean preparations of high soy protein content may not be conducive to equol production; however, the plasma levels of daidzein in the S/D study were not less than the other studies and thus there should have been equivalent opportunity for equol production. In the S/PB study, the proportion of subjects with plasma equol was 33% after soy and control yoghurt intake, 67% after both soy and probiotic yoghurt intake and control soy intake and 50% after soy and RS-enriched bread intake. In the S/O study, 67% of subjects had plasma equol after concurrent olive oil

supplementation and 39% after concurrent DHA oil supplementation. Thus, there may have been effects of these other dietary components on equol production, however further investigation is necessary to overcome some of the limitations due to large variability.

Overall, the highest plasma equol levels and prevalence were in the S/O study in which the soy food provided a higher level of glycitein and lower amount of genistein. The metabolites of glycitein are not well established, however Heinonen and co-authors (2003) identified glycitein metabolites in human urine after soy consumption and reported common end products of metabolism of daidzein and glycitein, one of which, 4',6,7-trihydroxyisoflavone differs to equol only in the presence of an extra hydroxyl group. The S/O study provides further evidence suggesting that increased metabolism to equol may occur when high levels of glycitein are consumed. Another possibility is that the close structural similarity between this glycitein metabolite and equol may have caused their co-elution in the current HPLC-ECD system used. The standard for this isoflavone would be necessary to determine whether this was true. Alternatively, perhaps reduced genistein content of soy food allows more daidzein metabolism if these two isoflavones are acted on by the same bacteria and compete for available enzymes. It also seems likely that daidzein and glycitein may undergo similar metabolic reactions different to those of genistein, as the latter is unique in having a hydroxyl group adjacent to the carbonyl moiety on ring A (Figure 1.1). Hydrogen bonding between these two functional groups has been suggested to reduce reactivity of genistein (Coldham *et al.*, 2002).

Thus, overall none of the dietary treatments with soy in the studies of this thesis were able to significantly and consistently enhance the metabolism of daidzein to equol, but again highlighted the large inter-individual variability in this process. Although there were high correlations between daidzein and genistein levels, further metabolism of these compounds seems dependent on other factors. However, there was a trend in the S/PB study for equol-negative subjects to have the lowest plasma levels of daidzein and genistein, suggesting that metabolism of daidzein to equol may be dependent initially on

the amount absorbed. Watanabe and Adlercreutz (1998) reported that equol was not associated with other isoflavones and that differences in the occurrence of this isoflavone metabolite were attributable to differences in hepatic enzymes and/or intestinal bacterial flora. However, neither probiotic nor prebiotic treatment in the S/PB study, which would have altered gut microflora, significantly affected equol production, although there was a trend of increased plasma equol with probiotic intake. Therefore, these gut microfloral attributes may not be modifiable by diet. In a study by Akaza and co-authors (2004), more than 45% of Japanese and Korean subjects, but only 14% of their American counterparts were able to produce equol, suggesting that genetics may play an important role. This was also suggested in the S/RS study in this thesis, in which two Kenyan subjects produced the highest amounts of equol.

7.4 Gender Differences in Isoflavone Bioavailability

In each of the three studies reported here, there were gender differences in isoflavone bioavailability, however there were no consistent trends between the studies. In the S/PB study, females had significantly higher concentrations of plasma daidzein than males at week 0 and there was a trend for consistently higher levels of plasma isoflavones in females during the study; in the S/O study there was a trend for males to have higher plasma genistein and equol but for females to have higher plasma daidzein after soy intake; and in the S/D study, males had significantly greater plasma daidzein and genistein after soy. However, there were no significant differences between genders in urinary isoflavone excretion, implying the isoflavone absorption and patterns of tissue distribution are different for females and males.

Other studies have reported gender differences in isoflavone bioavailability (Lu and Anderson, 1998; Zhang *et al.*, 1999), phytoestrogen lignan metabolism (Kirkman *et al.*, 1995) and colonic function, including differences in transit time, fecal bulk, and bile acid excretion (Kirkman *et al.*, 1995). Thus, it is possible that the genders may also respond differently to the different dietary treatments in addition to differences in isoflavone

bioavailability. Further, Coldham and Sauer (2000) reported gender differences in rats after administration of radioactive genistein, with female rats having higher levels in the liver. These authors suggested that there was greater isoflavone retention in the liver of females than males and also reported higher levels of genistein in reproductive tissues than other peripheral organs, supporting the idea that distribution may be related to estrogen receptor distribution and density. Maubach and co-authors (2003) suggested that isoflavones in tissue could occupy estrogen receptor sites or accumulate in lipophilic tissues. The additional findings in the S/PB study of a significant inverse correlation between body mass and plasma daidzein in all subjects suggests that greater body mass and potentially more adipose may promote higher tissue and lower plasma isoflavone levels. The significant positive correlation between age and plasma daidzein in females in the S/PB study suggests that in postmenopausal women, endogenous estrogen levels or estrogen receptor densities may further influence isoflavone bioavailability. Maubach and colleagues (2003) determined isoflavone levels in tissues and found a predominance of equol compared with daidzein and genistein with concentrations much higher than in serum (>1000 ng/mL). It is not known whether conversion of daidzein to equol is possible at target tissues or whether equol preferentially partitions into tissues rather than plasma. Further investigation of tissue distribution of isoflavones and of relations between isoflavone bioavailability and endogenous hormones and estrogen receptor density may aid in the understanding of their physiological mechanisms of action.

7.5 Lipid Effects with Soy Intake

All of the soy treatments from the S/D, S/PB and S/O studies, despite differences in soy protein and individual isoflavone content (Table 7.1), resulted in mean decreases in TCh and LDL (Table 7.3). However, decreases in TCh were only significant for the dietary combination of soy with a probiotic or prebiotic (S/PB study) and decreases in LDL were only significant for the control soy and prebiotic treatments in the S/PB study. Mean baseline lipid levels were highest in the S/PB study and lowest in the S/D study; however, the mean changes in TCh, LDL and HDL were similar for the three studies

(Table 7.3). The smallest decrease in LDL was in the S/O study when soy was combined with DHA, demonstrating the effects of the DHA-rich oil in increasing LDL.

The only mean reductions in TG and increases in HDL were in the S/D study and after combined intake of soy and DHA-oil intake in the S/O study (attributed to the DHA-oil, Chapter 6). The trend for decreased TG levels and increased HDL levels with the S/D study may support evidence suggesting that soy protein is necessary for these lipid benefits. Alternatively, these effects may be due to the α -linolenic content of the whole soy bean extract, as this fatty acid is a precursor to DHA and EPA, although endogenous conversion is limited (Harris, 1997). Furthermore, the greatest mean increases in TG levels were with the soy controls in both the S/PB and S/O studies, suggesting that increases in TG with soy intake may be somewhat counteracted by concurrent soy protein, n-3 fatty acid, probiotic or prebiotic intake. Although it is possible that soy protein may have contributed to the decreases in TG and increases in HDL in the S/D study, comparison between the three studies in this thesis suggests soy protein is not necessary for, nor does its absence preclude, reductions in TCh or LDL.

The lipid changes reported in these three studies are of a similar magnitude to those reported in two recent meta-analyses of studies that used similar intakes of soy protein and isoflavones in subjects with baseline total cholesterol between 4.57 and 6.60 mmol/L (Harland and Carr, 2004; Weggemans and Trautwein, 2003) and within the range of other published results (Table 7.4). There did not appear to be a clear trend between soy protein or isoflavone levels and lipid changes in the three studies in this thesis. This is in agreement with the meta-analysis by Weggemans and Trautwein (2003) and other studies (Table 7.4) in which dose-dependent effects of isoflavones are sometimes but not always reported. However, isoflavones independently do not usually result in significant lipid-beneficial effects (Hodgson *et al.*, 1998; Nestel *et al.*, 1997), as confirmed in a recent meta-analysis (Yeung and Yu, 2003). The similar reductions in TCh and LDL in the S/D, S/PB and S/O studies in which the daily intake of soy protein was 30 g, 10 g and less than 2 g, respectively, suggest some lipid-lowering ability of the isoflavones alone and not necessarily dependent upon soy protein intake.

Table 7.3 Mean baseline lipids and lipid effects of soy intake for thesis studies.

		Ch 4	Ch 5				Ch 6	
Total isoflavones		82 mg	110.5 mg				89.5 mg	
Soy protein		30 g	10 g				< 2 g	
Dietary combination		soy	control yoghurt	probiotic yoghurt	control	resistant starch	olive oil	DHA oil
n		23	10	6	9	6	15	11
baseline	TCh	5.70 ± 0.23	6.74 ± 0.32	6.70 ± 0.30	6.39 ± 0.18	6.62 ± 0.15	6.04 ± 0.24	6.29 ± 0.21
	LDL	3.99 ± 0.24	4.64 ± 0.33	4.52 ± 0.30	4.22 ± 0.19	4.44 ± 0.17	4.04 ± 0.19	4.27 ± 0.22
	HDL	1.14 ± 0.07	1.23 ± 0.10	1.24 ± 0.09	1.29 ± 0.07	1.29 ± 0.07	1.13 ± 0.07	1.18 ± 0.07
	TG	1.30 ± 0.14	1.99 ± 0.32	2.05 ± 0.23	1.93 ± 0.17	1.97 ± 0.15	1.91 ± 0.29	1.84 ± 0.22
change	TCh	-0.14 ± 0.15 (-1.6 ± 2.6%)	-0.12 ± 0.12 (-1.5 ± 1.7%)	-0.32 ± 0.14 (-4.7 ± 2.0%)	-0.09 ± 0.08 (-1.1 ± 1.8%)	-0.36 ± 0.10 (-5.5 ± 1.6%)	-0.07 ± 0.15 (-1.1 ± 2.4%)	-0.11 ± 0.11 (-1.4 ± 1.7%)
	LDL	-0.11 ± 0.13 (-1.4 ± 3.5%)	-0.14 ± 0.10 (-2.5 ± 2.6%)	-0.17 ± 0.10 (-3.9 ± 2.4%)	-0.21 ± 0.09 (-4.1 ± 2.1%)	-0.32 ± 0.10 (-7.3 ± 2.2%)	-0.20 ± 0.12 (-4.8 ± 2.9%)	-0.06 ± 0.12 (-0.3 ± 3.0%)
	HDL	+0.04 ± 0.05 (4.7 ± 4.1%)	-0.01 ± 0.03 (-0.3 ± 2.8%)	-0.09 ± 0.05 (-6.5 ± 3.9%)	-0.02 ± 0.03 (-1.7 ± 2.5%)	-0.04 ± 0.03 (-2.1 ± 2.4%)	-0.01 ± 0.03 (0.8 ± 3.3%)	+0.03 ± 0.02 (2.4 ± 1.9%)
	TG	-0.16 ± 0.08 (-4.1 ± 5.8%)	0.21 ± 0.21 (22.3 ± 10.7%)	0.03 ± 0.15 (2.0 ± 6.0%)	0.29 ± 0.19 (17.2 ± 10.0%)	0.01 ± 0.15 (4.8 ± 7.7%)	0.31 ± 0.31 (16.0 ± 10.8%)	-0.19 ± 0.13 (-7.4 ± 5.7%)

Table 7.4 Comparison of lipid effects reported in the literature.

Reference	Subjects	Duration	Isoflavones (mg/day)	SP (g/day)	Baseline (mmol/L)		Lipid effects
					TCh	LDL	
(Gooderham <i>et al.</i> , 1996)	20 M	4 wk II	36 D + 80 G + 15 g	43.5	4.4		none
(Steinberg <i>et al.</i> , 2003)	28 F (post)	6 wk X	108 total	25	4.9	2.9	none
(Kreijkamp-Kaspers <i>et al.</i> , 2004)	202 F (post)	12 m II	41 D + 52 G + 6 g	26	6.2	4.2	none
(Mitchell and Collins, 1999) [†]	10 M	4 wk II	?	?	4.6		none
(Merz-Demlow <i>et al.</i> , 2000)	13 F (pre)	3 m X	10 vs. 65 vs. 129	53	3.9	2.3	65 mg isos SIG ↓ LDL (~9%)
(Tonstad <i>et al.</i> , 2002)	130 M + F (post)	16 wk II	110 vs. 180	30 50	6.7	4.8	SP >↓ TCh (-0.24 mmol/L) SP >↓ LDL (-0.26 mmol/L) No dose-dependent effects of isos
(Gardner <i>et al.</i> , 2001) **	94 F (post)	12 wk II	25 D + 52 G + 4 g	42	6.2	4.2	SP + isos >↓ TCh (-0.27 mmol/L) SP + isos >↓ LDL (-0.38 mmol/L)
(Baum <i>et al.</i> , 1998)	66 F (post)	6 m II	18 D + 34 G + 4 g 33 D + 49 G + 8 g	40	6.5		SP ↓ TCh/HDL No dose-dependent effects of isos
(Bricarello <i>et al.</i> , 2004) [†]	60 M + F (pre + post)	6 wk X	33 D + 50 G + 5 g	25	6.2	4.1	SM SIG ↓ LDL (-0.31 mmol/L) SM SIG ↑ HDL (+0.10 mmol/L)
(Potter <i>et al.</i> , 1998)	66 F (post)	24 wk II	56 vs. 90	40	6.5		SP ↓ TCh (-0.37 mmol/L) 90 mg isos SIG ↑ HDL 56 mg isos SIG ↓ TCh/HDL
(Crouse <i>et al.</i> , 1999)	156 M + F (pre + post)	9 wk II	3 vs. 27 vs. 37 vs. 62	25	6.3	4.3	62 mg isos SIG ↓ TCh (-0.25 mmol/L) 62 mg isos SIG ↓ LDL (-0.26 mmol/L)
(Cuevas <i>et al.</i> , 2003)	18 F (post)	4 wk X	24 D + 48 G + 8 g	40	7.4	5.0	SP and C both SIG ↓ TCh and LDL SP SIG ↓ TG (-0.6 mmol/L)
(Lichtenstein <i>et al.</i> , 2002) **	42 M + F (post)	6 wk X	40 D + 70 G + 10 g	50	6.2	4.1	SP SIG ↓ TCh and LDL No effect isos
(Wangen <i>et al.</i> , 2001)	18 PM	3 m X	7 vs. 65 vs. 132	63	5.6	3.5	SP + isos SIG ↓ TCh and LDL Dose-dependent effect of isos

[†] Compared soy milk vs non-fat cows milk. All others used soy protein compared with either casein or milk protein.

M = male. F = female. Pre = premenopausal. Post = postmenopausal. C = control. > = more than control.

** compared with and without isoflavones as well as control. D = daidzein, G = genistein, g = glycitein.

II – parallel study design, X = crossover study design.

In the three studies of this thesis examining lipid measures, there were significant inverse correlations between baseline TCh and the reductions of TCh with soy intake. There appeared to be differences in lipid effects between those with baseline TCh either greater or less than 6.0 mmol/L. Similarly, there was a trend in the studies presented in Table 7.4 for a relationship between greater lipid-beneficial effects and baseline TCh levels greater than 6 mmol/L. Consequently, subjects in the studies here were classified according to their baseline TCh levels. The only significant reduction in TCh with control soy treatment was in subjects with TCh greater than 6.0 mmol/L in the S/D study (Table 7.5). In the S/PB study, there was a significant decrease in TCh in subjects with baseline TCh greater than 6.0 mmol/L with prebiotic treatment; however probiotic treatment showed a trend towards a reduction in TCh independent of baseline TCh, suggesting it may have promoted broader lipid-lowering effects. There was also a near significant reduction in TCh with soy plus DHA intake in the S/O study. Therefore, baseline TCh has some influence on the lipid-lowering effects of soy.

Table 7.5 Influence of baseline TCh on lipid effects of soy treatments.

Study	Treatment	Baseline TCh	n	Baseline TCh	Change (mmol/L)	Change %	p
S/D	soy	< 6	15	5.11 ± 0.16	0.12 ± 0.16	2.7 ± 3.1	0.476
		> 6	8	6.76 ± 0.25	-0.63 ± 0.21	-9.7 ± 3.2	0.019
S/PB	soy control	< 6	7	5.51 ± 0.11	0.06 ± 0.14	1.1 ± 2.4	0.058
		> 6	23	6.88 ± 0.06	-0.16 ± 0.10	-2.1 ± 1.6	0.691
	probiotic	< 6	4	5.52 ± 0.12	-0.23 ± 0.13	-4.1 ± 2.4	0.176
		> 6	11	7.13 ± 0.32	-0.35 ± 0.18	-4.9 ± 2.6	0.088
	prebiotic	< 6	3	5.62 ± 0.05	-0.09 ± 0.09	-1.6 ± 1.5	0.405
		> 6	12	6.85 ± 0.09	-0.42 ± 0.12	-6.3 ± 1.9	0.005
S/O	SC + OO	< 6	9	5.45 ± 0.13	-0.10 ± 0.08	-1.8 ± 1.4	0.219
		> 6	6	6.93 ± 0.33	-0.03 ± 0.38	-0.05 ± 5.9	0.934
	SC + DHA	< 6	8	5.52 ± 0.11	0.09 ± 0.13	1.8 ± 2.4	0.521
		> 6	10	6.90 ± 0.21	-0.28 ± 0.14	-4.0 ± 2.1	0.084

There were also significant inverse associations between baseline LDL and subsequent change in LDL with soy intake for S/D, S/PB and S/O studies. When subjects were divided according to whether their baseline LDL was less than or greater than 4.0

mmol/L, there was a trend for a greater reduction in those with LDL greater than 4.0 mmol/L with all dietary treatments compared with either a smaller reduction or an increase in LDL in those with initial LDL levels greater than 4.0 mmol/L (Table 7.6). However, this was not significant for all treatments. A similar relationship between baseline LDL and subsequent reductions in LDL with soy intake has also been reported in other studies (Crouse *et al.*, 1999).

Table 7.6 Influence of baseline LDL on lipid effects of soy treatments.

Study	Treatment	Baseline LDL (mmol/L)		n	Change (mmol/L)	Change (%)	p
			mean				
S/D	soy	<4.0	3.29 ± 0.15	13	0.09 ± 0.17	3.1 ± 5.3	0.605
		>4.0	4.81 ± 0.23	10	-0.36 ± 0.18	-7.3 ± 3.5	0.077
S/PB	S + YC	<4.0	3.54 ± 0.16	8	0.12 ± 0.21	3.7 ± 5.9	0.613
		>4.0	5.03 ± 0.38	2	-0.24 ± 0.11	-4.8 ± 2.6	0.057
	S + YP	<4.0	3.58 ± 0.10	8	-0.13 ± 0.14	-3.3 ± 3.9	0.382
		>4.0	5.35 ± 0.36	2	-0.20 ± 0.16	-4.5 ± 3.2	0.259
	SC	<4.0	3.39 ± 0.16	12	0.21 ± 0.09	6.2 ± 2.9	0.115
		>4.0	4.53 ± 0.19	3	-0.36 ± 0.07	-7.9 ± 1.5	0.001
	S + RS	<4.0	3.44 ± 0.21	6	-0.13 ± 0.10	-3.5 ± 2.9	0.328
		>4.0	4.66 ± 0.13	10	-0.37 ± 0.12	-8.2 ± 2.6	0.013
S/O	SC + OO	<4.0	3.77 ± 0.21	10	-0.15 ± 0.10	-3.7 ± 2.8	0.225
		>4.0	4.58 ± 0.23	5	-0.32 ± 0.32	-6.9 ± 7.4	0.274
	SC + DHA	<4.0	3.56 ± 0.11	8	0.31 ± 0.12	8.7 ± 3.4	0.040
		>4.0	4.85 ± 0.26	10	-0.35 ± 0.14	-7.5 ± 3.1	0.036

Earlier reviews also reported a greater decrease in TCh and LDL cholesterol after soy protein consumption in hypercholesterolemic people relative to normocholesterolemic subjects (Carroll, 1991; Carroll and Kurowska, 1995) and a significant influence of initial cholesterol levels was also reported in the original meta-analysis of soy protein and lipids (Anderson *et al.*, 1995). However, Wong and co-authors (1998) reported no differences in the effects of soy protein on lipids between normocholesterolemic and hypercholesterolemic men, and other studies have demonstrated lipid-lowering effects of soy intervention in subjects that did not have elevated baseline lipid levels (Merz-Demlow *et al.*, 2000; Nilausen and Meinertz, 1998). Therefore, although initial lipid

levels may influence to a certain extent the strength of lipid effects of soy intake, it appears that a number of factors are involved.

7.6 Conclusions

In four different soy isoflavone bioavailability studies in humans, large inter-individual variability was observed. Nonetheless, soy intake significantly increased plasma and urinary isoflavone levels which was dependent to a certain degree on the isoflavone levels in the food. In the first study (S/RS), the intake of resistant starch in the same meal as soy decreased isoflavone bioavailability but resistant starch consumption for 2 – 4 weeks increased daidzein excretion and equol production in some subjects, particularly in two Kenyan subjects. This finding led to the hypothesis that prebiotic intake may enhance isoflavone bioavailability, which was tested in the third study (S/PB). However, in this larger study of men and postmenopausal women, neither probiotic nor prebiotic intake significantly affected soy isoflavone bioavailability. Although there were some trends, including increased plasma equol after probiotic intake, it was concluded that individual factors in relation to isoflavone bioavailability may be more important. In the S/RS study, the increased equol production in the Kenyan subjects may have been due to different genetics or gut microflora profiles, suggesting these are key components in the determination of equol production.

Differences in isoflavone bioavailability were observed depending on the matrix and isoflavone composition of the soy foods. Soy milk intake in the second study (S/D) appeared to result in increased genistein bioavailability, while in the last study (S/O), consumption of soy cereal with reduced genistein content resulted in significantly less plasma and urinary genistein. There were also differences in equol prevalence between the studies, although overall, there was no outstanding factor to which equol production could be attributed to.

As a clinically relevant outcome measure, lipids were analysed and compared with soy isoflavone bioavailability. There were no associations between isoflavone bioavailability and lipid changes with soy consumption. Although equol-positive subjects in the S/D study displayed significant lipid-lowering effects, this may have been due to their higher mean baseline cholesterol levels. Overall, soy intake did not produce significant lipid lowering effects, except in subjects with the highest baseline levels. However, in combination with a probiotic or prebiotic, significant reductions in TCh were found, suggesting a health benefit with dietary combination of these foods. Further, in combination with DHA-rich oil, soy cereal attenuated increases in TCh and LDL that were observed with the DHA control, suggesting this may be another beneficial dietary combination.

In conclusion, plasma and urinary isoflavone levels were significantly elevated after soy intake in all studies of this thesis. It appears that the processes of isoflavone absorption and metabolism, including that of daidzein to equol, are highly variable between individuals and not easily modifiable by dietary interventions including those using probiotic or prebiotics. However, there were some significant effects and trends reported in this thesis that are interesting and worth examining further. It would be beneficial to further investigate the potential for probiotic and prebiotic intake to increase isoflavone bioavailability as this may provide some insight into favourable gastrointestinal characteristics for enhanced isoflavone bioavailability and metabolism to equol. Determination of gut microflora types prior to and after probiotic or prebiotic intake in relation to isoflavone bioavailability would be useful in identifying those bacteria that are involved in this process and also to identify particular microfloral profiles that are responsive to such treatments. Cross-population studies may also help establish the role of genetics and/or the effect of habitual diet on dietary modifications and soy isoflavone bioavailability as suggested in Chapter 3. Furthermore, comparisons of isoflavone bioavailability between premenopausal and postmenopausal women or between women who have been postmenopausal for varying lengths of time in combination with measures of endogenous hormone levels may assist in understanding the role of gender and possibly estrogen receptors in determining isoflavone bioavailability. All such

studies should include clinically relevant measures such as lipids or of oxidation status since, ultimately, a link between isoflavone bioavailability and physiological effects and/or health benefits is desirable.

CHAPTER 8

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