BIFIDOBACTERIAL ENZYMIC TRANSFORMATION OF ISOFLAVONES, THEIR BIOAVAILABILITY AND EFFECTS ON DISEASE RISK BIOMARKERS IN POSTMENOPAUSAL WOMEN

A thesis submitted for the degree of Doctor of Philosophy

By

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I, the first son of Vasilios, would like to dedicate this thesis comprising my first contribution to science to the memory of my beloved father who was the victim of acute myeloid leukaemia in 1986 at the age of 35 years. His memory and metaphysical presence in my life has been my greatest driving force in the past few years since discovering my Self and the pathway in life I would like to follow. Secondly, I would like to acknowledge the most important living figure in my life, my determined, self-reliant and courageous mother. Her sole dedication in life to raising her children is greatly appreciated.

Abstract

Soybeans and non-fermented soyfoods, including soymilk, contain only 5 to 10% of total isoflavone in a biologically active aglycone configuration, with the remainder found as isoflavone glucoside conjugates. Saccharolytic enzyme-producing *Bifidobacterium*, classified as probiotics, are commonly incorporated into fermented milk products to enhance their health benefit. Postmenopausal women, who are associated with high rates of chronic disease due to ovarian failure and subsequent diminishing oestrogen levels, are seeking natural alternatives to oestrogen and hormone replacement therapy due to its proposed adverse cancer-related effects. The main objectives of this study were to develop an isoflavone aglycone-enriched fermented soymilk containing viable bifidobacteria and examine the effects of ingesting this product on the bioavailability of isoflavone and markers of disease risk in postmenopausal women.

Five strains of *Bifidobacterium* were screened for β-glucosidase activity using p-nitrophenyl-β-D-glucopyranoside as the substrate. Selected strains were used to ferment soymilk made from Supro® soy protein isolate 590 (SPI590). Enumeration of viable *Bifidobacterium* populations, pH measurements, and quantification of isoflavones using HPLC were performed at 0, 12, 24, 36 and 48 h of incubation. Four strains of bifidobacteria produced β-glucosidase. *Bifidobacterium pseudolongum* (BP20099) and *Bifidobacterium longum* BB536 (BB536) displayed the best growth in soymilk, with an increase of 1.3 $\log_{10}$ CFU per mL after 12 h of incubation. *Bifidobacterium animalis* Bb-12 (BB12), BB536 and BP20099 caused enzymic hydrolysis of isoflavone malonyl-, acetyl- and β-glucosides to form aglycones. Fermentation of soymilk with *Bifidobacterium* sp. significantly increased the concentration of bioactive isoflavone aglycone from approximately 8% (at 0 h) to 50% of total isoflavone after 24 h of incubation ($P<0.05$).

In an attempt to enhance the biotransformation of isoflavone glucosides into aglycone in soymilk by *Bifidobacterium* sp., the same fermentation studies (described above) were then carried out on supplemented soymilk prepared by reconstituting SPI590 with the addition of 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine. Supplementation of soymilk did not significantly enhance the growth

of *Bifidobacterium* sp. between 0 and 12 h of incubation ($P>0.05$); however, bacterial growth improved between 12 and 48 h of incubation. The increase in the concentration of isoflavone aglycone caused by the biotransformation of isoflavone glucosides was significantly lower in supplemented soymilk after 24 h of fermentation with *Bifidobacterium*, when compared to plain soymilk ($P<0.05$). Results indicated that soymilk does not require supplementation with D-glucose and L-cysteine to enhance the biotransformation of isoflavone glucosides into bioactive forms.

Samples of non-fermented and fermented soymilk, with and without D-glucose and L-cysteine, collected at 0, 12, 24, 36 and 48 h of incubation, were then analysed for their content of α-galactosyl oligosaccharides, organic acids and aldehydes. In addition, four strains of *Bifidobacterium*, selected for their β-glucosidase activity, were also assessed for α-galactosidase activity using p-nitrophenyl-α-D-galactopyranoside as the substrate. In soymilk, oligosaccharides and aldehydes were effectively metabolised by *Bifidobacterium*. D-glucose and L-cysteine supplementation enhanced the hydrolysis of raffinose and stachyose. BB536 and BP20099 degraded a significantly greater level of oligosaccharides and aldehydes than BB12 and *Bifidobacterium longum* 1941 ($P<0.05$). In plain soymilk, hexanal and pentanal were not detected after 12 h of fermentation with BB536 and BP20099. Raffinose and stachyose were completely metabolised by BB536 after 48 h. BB536 also exhibited the highest α-galactosidase activity in the presence of D-raffinose, followed by BP20099 ($P>0.05$). Furthermore, BB536 was the highest producer of organic acids, with an average acetic acid/L (+)-lactic acid ratio of 0.7.

From enzyme assays and soymilk fermentation studies, BB12 exhibited the highest β-glucosidase activity and hydrolysed the greatest concentration of isoflavone glucosides into aglycone, respectively. Hence, this strain of bifidobacteria was chosen for the development of an isoflavone aglycone-enriched fermented soymilk. Three soymilk formulations were prepared by reconstituting Supro® soy protein isolate 159 (SPI159) and soy germ (SG) at ratios of 9:1 (SPI/SG9:1), 6:4 (SPI/SG6:4) and 3:7 (SPI/SG3:7), with each fermented by BB12. Enumeration of viable BB12 populations, pH measurements, and quantification of isoflavones were performed at 12 and 24 h of

incubation and at 1, 7 and 14 days of refrigerated storage. Isoflavone concentrations were 3, 7 and 12 times higher in SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7 than conventional soymilk made from SPI and BB12 populations increased respectively by 1.2, 1.3 and 0.9 $\log_{10}$ CFU per mL after 24 h to reach levels that exceeded 8 $\log_{10}$ CFU per mL. Hydrolysis of isoflavone glucosides increased the concentration of aglycone from 0.82 to 5.65 mg per 100 mL (47% of total isoflavone) in SPI/SG9:1 after 24 h ($P<0.05$), with isoflavone biotransformation less apparent in SPI/SG3:7. Fermented SPI/SG6:4 contained the highest concentration of isoflavone aglycone at 7.80 mg per 100 mL (26% of total). Viability of BB12 and composition of isoflavones were unchanged after 14 days refrigerated storage ($P>0.05$).

The effects of ingesting an isoflavone aglycone-enriched fermented soymilk on urinary isoflavone excretion and percentage recovery was then investigated in postmenopausal women. Sixteen postmenopausal women were randomly divided into two groups to consume either fermented or non-fermented soymilk. Each group participated in a double-blind, crossover study with three 14-day supplementation periods, separated by a 14-day washout. Subjects ingested three daily doses of isoflavone via the soymilk and collected four 24-hour pooled urine specimens per supplementation period. Isoflavone isomers and daidzein metabolite equol were quantified using HPLC. Non-fermented soymilks at 20, 40 and 80 mg isoflavone per 200 mL contained 10%, 9% and 7% aglycone, respectively, with their fermented counterparts containing 69%, 57% and 36% aglycone ($P<0.001$). A trend to greater urinary percentage recovery of daidzein and glycitein was observed amongst women consuming fermented soymilk at a dosage of 40 mg isoflavone ($P=0.13$). There was a distinct linear dose response for the fermented soymilk group ($R^2=0.9993$) compared to the non-fermented group ($R^2=0.8865$), suggesting less inter-individual variation in isoflavone absorption. However, concentrations of total isoflavone excreted in urine were similar for both groups ($P>0.05$), with urinary isoflavone recovery at approximately 31%. Furthermore, mean urinary excretion and percentage recovery of equol by the fermented soymilk group was not significantly greater than that of the non-fermented soymilk group ($P>0.05$), even though four of the six women classified as equol producers (that is, excreted >1 μmol equol per day) were from the fermented soymilk group. On the whole, according to the isoflavone profiles excreted in urine, there

was no strong evidence to indicate that ingesting soymilk fermented by bifidobacteria enhances the bioavailability of isoflavone compared to an equivalent non-fermented soymilk.

A clinical study was then carried out to examine the effects of ingesting an isoflavone aglycone-enriched fermented soymilk on hormone profiles and risk factors associated with cardiovascular disease and osteoporosis in postmenopausal women. Thirty-six healthy postmenopausal women, randomly allocated to one of three groups, participated in a double-blind, placebo-controlled, parallel study involving a 2-week baseline period followed by a 12-week supplementation period. Two groups, each containing 13 subjects, consumed 400 mL of either fermented soymilk or non-fermented soymilk on a daily basis providing 80 mg isoflavone per day. The fermented soymilk comprised ~57% of total isoflavone in an aglycone form, whereas its non-fermented counterpart contained ~9% aglycone. In addition, fermented soymilk provided viable populations of BB12 at levels of 10^8 viable cells per mL. The placebo group (n=10) consumed 400 mL of casein-milk per day during supplementation. Blood and spot urine specimens were collected from each subject at baseline and at week 12 of supplementation. Levels of follicle-stimulating hormone (FSH), luteinising hormone (LH), sex hormone binding globulin (SHBG), lipid profiles, and lipoprotein(a) [Lp(a)] were analysed in serum. Bone resorption marker deoxypyridinoline (DPD) was analysed in urine. There was a trend toward a reduction in LH (10 to 20%) and FSH (5 to 9%) and an increase in SHBG (6 to 8%) in women consuming fermented and non-fermented soymilk, but these hormonal changes were not significantly different to those occurring in the placebo group (P>0.05). Supplementation of fermented soymilk significantly increased HDL-cholesterol (~20%) compared to the placebo (P=0.01), but was not accompanied by reductions in total cholesterol, triglyceride or LDL-cholesterol (P>0.05). Furthermore, the decrease in Lp(a) observed in the fermented soymilk group (~8%) was not significantly different to the decreases in Lp(a) shown by the non-fermented and placebo groups (P=0.8). Nevertheless, the fermented soymilk group did show a large reduction in the urinary excretion of bone resorption marker DPD (~17%) compared to the increases in DPD excretion observed in the non-fermented and placebo groups (P=0.05). In conclusion, there was a lack of strong evidence to indicate that ingesting isoflavone aglycone-enriched fermented soymilk has an oestrogenic effect on hormone levels (LH, FSH and SHBG) and reduces the risk of

cardiovascular disease in postmenopausal women [in terms of lipid profiles and Lp(a)], but there was evidence to suggest that this product may prevent bone loss and subsequently reduce the risk of osteoporosis.

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CERTIFICATE

This is to certify that this thesis entitled "BIFIDOBACTERIAL ENZYMIC TRANSFORMATION OF ISOFLAVONES, THEIR BIOAVAILABILITY AND EFFECTS ON DISEASE RISK BIOMARKERS IN POSTMENOPAUSAL WOMEN" submitted by Dimitri Tsangalis in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Food Science and Nutrition at Victoria University is a record of genuine research work carried out by him from March 2001 to August 2004 under my personal guidance and supervision. Furthermore, this thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Principal Supervisor:  (Prof. N.P. Shah)

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List of Publications, Conference Presentations and Awards

REFERRED RESEARCH PAPERS

(1) Tsangalis D, Ashton JF, McGill AEJ & Shah NP (2002) Enzymic transformation of isoflavone phytoestrogens in soymilk by β-glucosidase-producing bifidobacteria. *Journal of Food Science* **67**, 3104-3113.

(2) Tsangalis D, Ashton JF, McGill AEJ & Shah NP (2003) Biotransformation of isoflavones by bifidobacteria in fermented soymilk supplemented with D-glucose and L-cysteine. *Journal of Food Science* **68**, 623-631.

(3) Tsangalis D & Shah NP (2004) Metabolism of oligosaccharides and aldehydes and production of organic acids in soymilk by probiotic bifidobacteria. *International Journal of Food Science and Technology* **39**, 541-554.

(4) Tsangalis D, Ashton JF, Stojanovska L, Wilcox G & Shah NP (2004) Development of an isoflavone aglycone-enriched soymilk using soy germ, soy protein isolate and bifidobacteria. *Food Research International* **37**, 301-312.

(5) Tsangalis D, Wilcox G, Shah NP & Stojanovska L (2004) Bioavailability of isoflavone phytoestrogens in postmenopausal women consuming soymilk fermented with bifidobacteria. *British Journal of Nutrition* (in press).

CONFERENCE PRESENTATIONS

(1) Tsangalis D, Ashton JF, McGill AEJ & Shah NP. Metabolism of oligosaccharides and biotransformation of isoflavones by bifidobacteria in soymilk. Oral presentation made at the Fermentation and Bioprocessing Conference held in Melbourne, Victoria, Australia, April 18-19, 2002.

(2) Tsangalis D, Ashton JF, McGill AEJ & Shah NP. Biotransformation of soy isoflavone phytoestrogens by probiotic bifidobacteria. Chaired oral poster presentation made at the 9th World Congress on Clinical Nutrition held in London, United Kingdom, June 24-26, 2002.

(3) Tsangalis D, Ashton JF & Shah NP. Development of an isoflavone aglycone enriched soymilk using soy germ, soy protein isolate and bifidobacteria. Oral presentation made at the Institute of Food Technologists Annual Meeting and Food Expo held in Chicago, United States, July 12-16, 2003.

(4) Tsangalis D & Shah NP. Bifidobacteria are not just probiotic. A poster presented at the 12th World Congress of Food Science and Technology held in Chicago, United States, July 16-20, 2003.

(5) Tsangalis D, Stojanovska L, Shah NP & Wilcox G. Bioavailability of isoflavone phytoestrogens from soymilk fermented with probiotic bifidobacteria. Oral presentation made at Nutrition Week held in Las Vegas, United States, February 9-12, 2004.

AWARDS

(1) **Third place winner of the Institute of Food Technologists George F. Stewart International Research Paper Competition 2003.** Selected as one of five finalists to present a paper on original research at the 2003 Institute of Food Technologists Annual Meeting and Food Expo in Chicago, United States, July 12-16, 2003. The paper was titled 'Development of an isoflavone aglycone enriched soymilk using soy germ, soy protein isolate and bifidobacteria' and this paper has been published in Food Research International as mentioned above.

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List of Abbreviations

AB	*Lactobacillus acidophilus* and *Bifidobacterium* sp.
ABT	*Lactobacillus acidophilus*, *Bifidobacterium* sp. and *Streptococcus thermophilus*
ANOVA	Analysis of variance
AUC	Area under the curve
BB12	*Bifidobacterium animalis* Bb-12 (VUP 13519)
BB536	*Bifidobacterium longum* BB536 (CSCC 5550)
BL1912	*Bifidobacterium infantis* (CSCC 1912)
BL1941	*Bifidobacterium longum* (CSCC 1941)
BMI	Body mass index (kg/m^2)
BP20099	*Bifidobacterium pseudolongum* (CSCC 1944)
C_{max}	Maximum concentration
CAS	Casein milk
CC391	Calcium caseinate (Alanate™ 391)
CFU	Colony forming units
CRE	Creatinine
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DPD	Deoxypyridinoline
ER	Oestrogen receptor
ERα	Oestrogen receptor α
ERβ	Oestrogen receptor β
ERT	Oestrogen replacement therapy
F6PPK	Fructose-6-phosphate phosphoketolase
FS	Fermented soymilk
FSH	Follicle-stimulating hormone
GC	Gas chromatography
HDL	High-density lipoprotein
HDPE	High-density polyethylene
HPLC	High performance liquid chromatography
HPLC-UV/VIS	High performance liquid chromatography with ultraviolet-visible detection
HRT	Hormone replacement therapy
ISTD	Internal standard

LDL	Low-density lipoprotein
LH	Luteinising hormone
Lp(a)	Lipoprotein(a)
MRS	de Mann Rogosa Sharpe
MRS-gluc	de Mann Rogosa Sharpe broth containing additional 1% (w/v) D-glucose
MRS-lac	de Mann Rogosa Sharpe broth containing additional 1% (w/v) lactose
MRS-raf	de Mann Rogosa Sharpe broth containing additional 1% (w/v) D-raffinose
MUS	Monounsaturated
NFS	Non-fermented soymilk
pNPαG	p-Nitrophenyl-α-D-galactopyranoside
pNPβG	p-Nitrophenyl-β-D-glucopyranoside
PUS	Polyunsaturated
%COV	Percentage coefficient of variation
RHHC	Rapid hydration hydrothermal cooking
RSM	Reconstituted skim milk
SAT	Saturated
SERM	Selective oestrogen receptor modulator
SG	Soy germ
SG-soymilk	Soymilk made from 4% (w/v) soy germ
SHBG	Sex hormone binding globulin
SPE	Solid-phase extraction
SPI	Soy protein isolate
SPI159	Supro® FXP-H-159 soy protein isolate
SPI590	Supro® 590 soy protein isolate
SPI-soymilk	Soymilk made from 4% (w/v) soy protein isolate-159
SPI/SG9:1	Soymilk containing 9 parts of soy protein isolate-159 to 1 part of soy germ (total of 4% w/v)
SPI/SG6:4	Soymilk containing 6 parts of soy protein isolate-159 to 4 parts of soy germ (total of 4% w/v)
SPI/SG3:7	Soymilk containing 3 parts of soy protein isolate-159 to 7 parts of soy germ (total of 4% w/v)
SPI/SG20	Soymilk containing 9.4 parts of soy protein isolate-159 to 0.6 parts of soy germ (total of 4% w/v), providing an isoflavone content of 20 mg per 200 mL
SPI/SG40	Soymilk containing 8.0 parts of soy protein isolate-159 to 2.0 parts of soy germ (total of 4% w/v), providing an isoflavone content of 40 mg per 200 mL
SPI/SG80	Soymilk containing 5.2 parts of soy protein isolate-159 to 4.8 parts of soy germ (total of 4% w/v), providing an isoflavone content of 80 mg per 200 mL

$T½_{abs}$	Apparent half-life of absorption
$T½_{elim}$	Apparent half-life of elimination
TG	Triglyceride
Total-c	Total cholesterol
UHT	Ultra high temperature
UV/VIS	Ultraviolet-visible

1.0 General Introduction

Before the 1990's, the intake of soy-based foods by Western peoples was negligible compared to people of Asian cultures, predominantly due to the lack of knowledge of the potential health benefits of soy products and because of the objectionable aroma and flavour and undesirable flatus activity associated with soy food ingestion. However, the popularity of soy foods has grown enormously over the past decade, mainly because of ever-expanding scientific literature on the potential health benefits of soybean constituents and to the development of 'new-age' soy foods (e.g. soy burgers, soy ice cream and soy yoghurt) and modification of traditional soy foods more suited to the taste preferences of the West. Much of this growth in soy food popularity can be attributed to the United States, with regard to both the research effort on the potential health benefits and to product innovation. In the US, the increasing consumer awareness of soy products as healthy food ingredients has been fueled by the final approval of the health claim for soy protein by the Nutrition Labelling and Education Act in October 1999 (Uzzan & Labuza, 2003). In a recent functional foods trend survey, manufacturers developing functional foods ranked soy proteins as the second key ingredient, after antioxidants, for growing importance expectations in their formulation efforts during the next 2 years (O'Donnell, 2003). The Australian food marketplace, strongly influenced by the food culture of the US, also currently has a diverse range of 'new-age' and traditional soy foods on supermarket shelves to satisfy rapidly growing consumer demand.

Although soy protein has been given the tick of approval by US Food and Drug regulatory bodies, a large number of studies have suggested that isoflavones are the most beneficial group of soy components. Isoflavones, found abundantly in soybeans, are a group of phytoestrogens or plant-derived compounds with a structural homology to human oestrogens. Soybean and soy food derived isoflavones are found as four chemical forms, including aglycones and their malonyl-, acetyl- and β-glucoside conjugates. The biologically active, oestrogen-like isoflavone isomers are the aglycone configurations of genistein, daidzein and glycitein (Setchell & Cassidy, 1999). Aglycone isomers are able to bind to oestrogen receptor sites and hence mimic the functions of endogenous oestrogens in the human body (Setchell, 1998). From reviews of epidemiological (Cassidy, 1996; Setchell,

1995) and small-scale clinical studies (Anthony, 2000; Kurzer, 2000; Munro *et al.* 2003) isoflavone consumption has been associated with a reduced risk of most hormone-associated health disorders prevalent in current Western civilisations. Asian populations, with their high intake of isoflavones (50 to 70 mg per day) from a staple diet of soy foods, are known to have the lowest incidence of osteoporosis, menopausal symptoms and mortality from cardiovascular disease and cancer (Nagata *et al.* 1998).

In soybeans and soy foods, isoflavones are mainly found as biologically inactive glucoside conjugates, typically comprising 80 to 95% of the total isoflavone concentration (Wang & Murphy, 1994; Tsukamoto *et al.* 1995; Franke *et al.* 1999; Murphy *et al.* 1999; King & Bignell, 2000). Furthermore, isoflavones in an aglycone configuration are the isomers which are absorbed through the intestinal epithelium of humans, according to a recent study by Setchell *et al.* (2002*a*). Studies on isoflavone bioavailability have shown that isoflavone aglycones are absorbed faster and in higher amounts than their respective glucosides in humans (Hutchins *et al.* 1995; Izumi *et al.* 2000). Intestinal bacteria play a key role in the bioavailability and bioactivity of isoflavones (Hendrich, 2002). Hence, the ingestion of a soy food providing an aglycone-rich source of isoflavone and which is able to modulate intestinal microbial balance may be of great health benefit to consumers in preventing chronic diseases.

Of the traditional soy foods available on the marketplace, soymilk is probably the most well known and widely accepted amongst Western peoples. Soymilk, the aqueous extract of soybeans, originated in Asia and is a nutritious beverage closely resembling dairy milk in appearance and composition (Liu, 1997). Western populations, who are accustomed to the taste of dairy milk, generally dislike the flavour profile of traditional soymilk because of its aftertaste, often described as 'beany'. The beany flavour of soymilk is predominantly due to the presence of aldehyde compounds hexanal and pentanal (Wilkens *et al.* 1967). Soymilk also contains oligosaccharides, raffinose and stachyose, which are indigestible and commonly associated with flatulence in humans (Rackis *et al.* 1970). Methods of soymilk preparation have evolved to utilise defatted, protein-enriched, and low carbohydrate soy materials (e.g. soy protein isolate) in an attempt to simplify the

manufacture of soymilk, improve its flavour profile, and to prevent flatulence (Shurtleff & Aoyagi, 1984).

Soymilk may contain between 3 and 17 mg total isoflavone per 100 g wet weight (Murphy *et al.* 1999; Franke *et al.* 1999; King & Bignell, 2000), with soymilk made from soy protein isolate generally comprising a lower level of isoflavone than soymilk made from soybeans (Hutabarat *et al.* 2001; Setchell & Cole, 2003). Of the total concentration of isoflavones in soymilk greater than 90% of the isomers exist as biologically inactive glucosidic forms (Murphy *et al.* 1999; Setchell & Cole, 2003). Thus, the development of an isoflavone-rich soymilk comprising a greater proportion of isoflavone isomers in a bioactive aglycone configuration, that is simple to manufacture, imparts less 'beany' flavour, and causes less flatus activity would be of interest to the soy food manufacturing industry (such as, Sanitarium Health Food Company).

Bifidobacteria are classified as probiotic bacteria, which are commonly defined as viable bacteria that exhibit a beneficial effect on the health of the host upon ingestion by improving the balance of its indigenous microflora (Gomes & Malcata, 1999). Due to their health promoting properties, bifidobacteria are widely used in dairy preparations containing live microbial culture (Shah, 2000). The genus *Bifidobacterium* is a predominant member of the intestinal microflora and produce numerous saccharolytic enzymes (Desjardins & Roy, 1990), including β-glucosidase (Tochikura *et al.* 1986) and α-galactosidase (Scalabrini *et al.* 1998) needed to hydrolyse isoflavone glucosides and α-galactosyl oligosaccharides, respectively. According to *in vitro* studies, soymilk is a nutritious growth medium for *Bifidobacterium* sp. (Kamaly, 1997; Chou & Hou, 2000; Hou *et al.* 2000).

Diminishing levels of oestrogen caused by ovarian failure, inadequate to maintain oestrogen-dependent tissues, are associated with high rates of chronic disease in postmenopausal women. Hormone replacement therapy (HRT) is widely used by women in their menopausal years (especially of Western populations) for relief of menopausal symptoms and to minimise the risk of cardiovascular disease and osteoporosis. However, many women have been turning to

phytoestrogens, due to growing evidence of their oestrogenic activity, as a natural alternative to HRT and oestrogen replacement therapy because of their undesirable side effects, such as increased risk of breast cancer (Brzezinski & Debi, 1999; Wade *et al.* 1999; Wagner *et al.* 2001; Rossouw *et al.* 2002). In the past decade, numerous small-scale clinical studies involving postmenopausal women have investigated whether or not isoflavones derived from soy foods influence hormone-dependent states (hormone and endogenous oestrogen levels) and risk factors associated with cardiovascular disease and osteoporosis, but results have been highly variable and inconclusive. The types of isoflavone isomers ingested (isoflavone aglycone versus isoflavone glucoside) and intestinal microbial composition (predominant populations of enzyme-producing *Bifidobacterium* and other probiotic genera) may hold the key to finding a clearer association between isoflavones and disease prevention in this high-risk group.

This study was aimed at achieving two major objectives. The first aim was to develop a novel fermented soymilk containing viable bifidobacteria, and the second aim was to examine the physiological effects of this product on postmenopausal women. We hypothesized that the β-glucosidase activity of *Bifidobacterium* sp. would biotransform isoflavone glucosides into aglycone isomers during the fermentation of soymilk. Furthermore, postmenopausal women ingesting fermented soymilk comprising a higher proportion of isoflavone in a free, bioactive aglycone configuration would show a greater clinical response (in terms of changes in hormone profiles and risk factors associated with cardiovascular disease and osteoporosis) than those women consuming a non-fermented, isoflavone glucoside-rich soymilk. Thus, the specific objectives of this project were to:

(1) screen strains of *Bifidobacterium* for β-glucosidase activity, examine the growth of the selected strains in soymilk, and quantify the levels of isoflavone glucosides and aglycones before and after fermentation;

(2) examine the α-galactosidase activity of the selected strains of *Bifidobacterium* and quantify the oligosaccharide, aldehyde and organic acid levels in soymilk before and after fermentation;

(3) formulate a probiotic soymilk comprising a rich source of isoflavones (predominantly in an aglycone configuration) and high populations of bifidobacteria ($>10^6$ viable cells per mL);

(4) study the effects of consuming a probiotic soymilk on the bioavailability of isoflavones in postmenopausal women, in terms of urinary isoflavone excretion and percentage recovery, and

(5) study the effects of the concomitant ingestion of isoflavones and bifidobacteria on bone turnover, cardiovascular disease risk factors [lipid profiles and lipoprotein(a)], and sex steroid hormones (follicle-stimulating hormone, luteinising hormone and sex hormone binding globulin) in postmenopausal women.

With respect to the contents of this thesis, Chapter 2.0 contains the review of literature, Chapters 3.0, 4.0 and 5.0 report on enzyme assays and studies on soymilk fermentation. Chapter 6.0 deals with the development of the probiotic soymilk and studies on its isoflavone levels and the viability of bifidobacteria. Chapters 7.0 and 8.0 deal with the bioavailability of isoflavones in postmenopausal women consuming probiotic soymilk, and Chapter 9.0 focuses on the effects of ingesting a probiotic soymilk on the hormonal status of postmenopausal women as well as effects on biomarkers of disease risk. Overall conclusions and future research direction are described in Chapter 10.0, and a list of references given in Chapter 11.0.

2.0 Review of Literature

2.1 Soybeans and soy foods

Soybeans have been an important part of Asian culture for more than a thousand years, both as a food and as a medicine (Messina, 1995). Soybean foods are typically divided into two categories: non-fermented and fermented. Traditional non-fermented soy foods include fresh green soybeans, whole dry soybeans, soy nuts, soy sprouts, whole-fat soy flour, soymilk and soymilk products including tofu, okara and yuba. There are also non-fermented soy ingredients which are widely used in soy food production, including soy protein isolate (~90% w/w protein), soy protein concentrate (~70% w/w protein), soy grits (~58% w/w protein), soy germ powder (~40% w/w protein) and soybean oil (Liu, 1997; Schryver, 2002). Over the past decade, numerous 'second-generation' or 'new-age' soy foods have been developed to appeal to the wider community, including soy hot dogs, soy ice cream, soy burgers, soy flour pancakes and many other products mimicking foods typically containing animal protein or dairy ingredients. Alternatively, there are fermented soy foods including tempeh, miso, soy sauces, natto and 'new-age' fermented tofu and soymilk products (e.g. soy yoghurt and soy-based cheeses). However, according to the scientific literature and products on the Australian marketplace, there have been no previous attempts to formulate an isoflavone aglycone-enriched, probiotic soymilk using modern soy ingredients like soy protein isolate and soy germ powder and fermentation by bifidobacteria.

2.1.1 The soybean (*Glycine max* L. Merr.): components and composition

Mature soybean seeds basically comprise three parts: the seed coat, the embryo and one or more food storage structures (Figure 2.1). The embryo contains two pieces of cotyledons that function as food reserve structures, and has three other parts including the radicle, hypocotyl and epicotyl. The seed coat is marked with a hilum or seed scar and its primary function is to protect the embryo from fungi and bacterial infection before and after planting (Liu, 1997). The hypocotyl radicle or germ, which comprises about 2% (w/w) of the soybean seed, is the most isoflavone-rich component of the soybean, with a total isoflavone concentration of 5.5 to 6 times higher than that in the cotyledons (expressed as mg per 100 g dry weight) (Kudou *et al.* 1991). Furthermore, glycitein and its

glucosidic isomers occur exclusively in the hypocotyl (Kudou *et al.* 1991). However, Tsukamoto *et al.* (1995) reported that of the total isoflavones in the soybean, 80 to 90% are located in the cotyledons, apparently due to cotyledons being the highest proportion in the seed (approximately 90% w/w of the soybean seed). With respect to macronutrient composition, dry soybeans typically contain 40% (w/w) protein, 20% (w/w) lipids (85% of which is unsaturated), 35% (w/w) carbohydrate and 5% (w/w) ash (including potassium, phosphorous, magnesium, sulfur, calcium, chloride and sodium) (Liu, 1997). Cotyledons contain the highest percentage of both protein and oil and since they are the major component in the whole soybean, their composition is very close to that of the whole soybean regardless of great compositional differences among other structural parts (Liu, 1997).

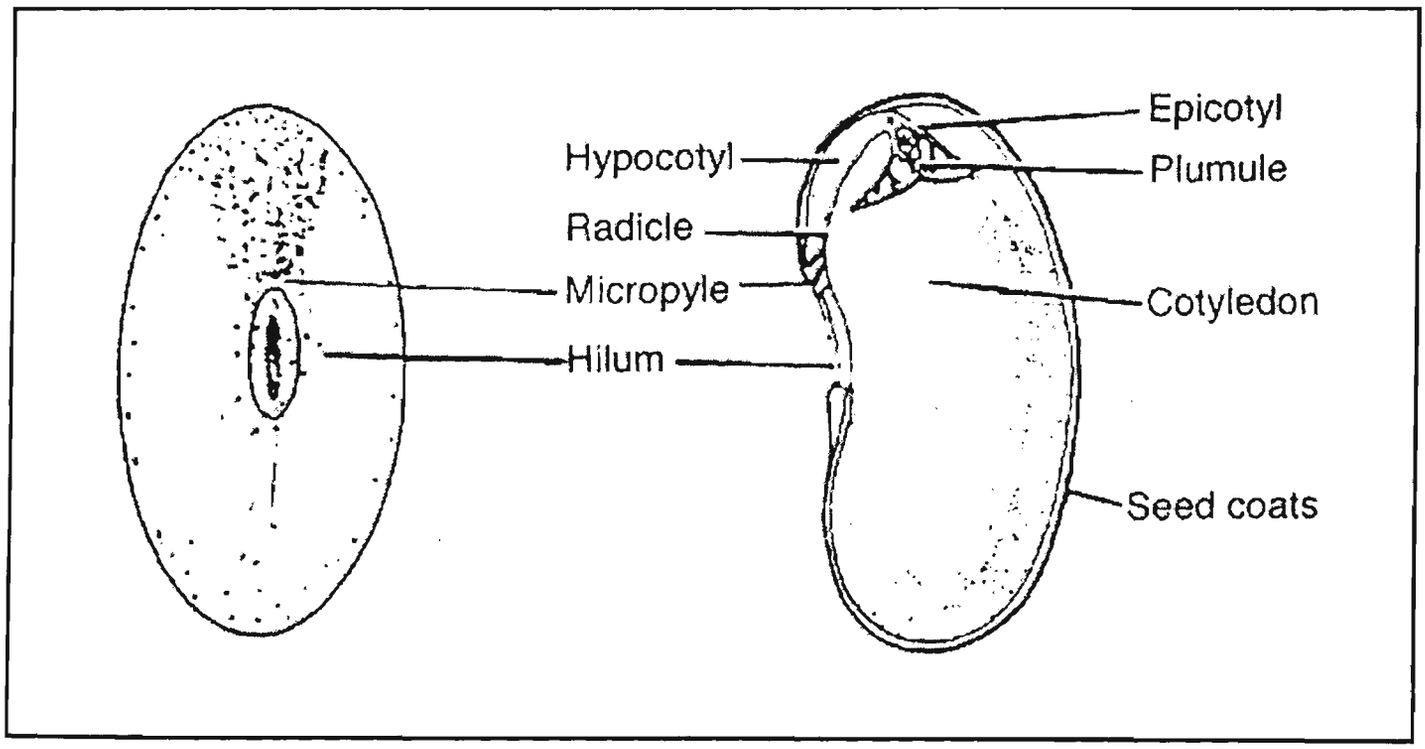

Figure 2.1 Structural components of a mature soybean seed. Sourced from Liu (1997).

Soybean protein is a major source of vegetable protein in the diet of Asian populations (Friedman & Brandon, 2001). According to protein digestibility corrected amino acid score (official assay used by the World Health Organisation and United States Food and Drug Administration), soy protein, with a scoring of 1.0, is considered to meet the protein needs of adults when consumed as the sole source of protein at the recommended level of intake (0.6 to 0.8 g per kg bodyweight) (Messina,

1995). However, soy protein is deficient in the essential amino acid methionine. Furthermore, adverse nutritional effects following the consumption of raw soybean meal have been attributed to the presence of endogenous inhibitors of digestive enzymes and lectins and to poor digestibility (Friedman & Brandon, 2001). These anti-nutritional components of soybeans are generally inactivated by heat treatment during processing to improve the nutritional quality of soy foods. Lastly, soybean foods are lactose and cholesterol free, low in saturated fats, and a good source of omega-3 fatty acids (Messina, 1995).

2.1.2 Soy protein isolate

Soy protein isolate (SPI) is generally commercially prepared from defatted soy meal (consisting of ground soy flakes which are prepared from soybeans by drying, cleaning, cracking, dehulling, flaking, defatting using hexane, and solvent removal) using aqueous or mild alkali extraction (pH 7 to 10) of proteins and soluble carbohydrates (Liu, 1997). The insoluble residue, mostly carbohydrate, is removed by centrifugation, followed by precipitation of soy protein at its isoelectric point (pH in the range of 4.5). The precipitated protein is then separated by mechanical decanting, washed and neutralized to a pH of 6.8 and then spray-dried. The resulting product is a highly purified proteinate form of soy protein (typically 90% w/w protein) with minimal 'beany' flavour (Waggle *et al.* 1989; Liu, 1997). The 'beany' flavour of soy foods is attributed to the oxidation of the lipid fraction (discussed in section 2.3) and the presence of isoflavones (discussed in section 2.5.4), both of which are at low levels in SPI. A brand of SPI which is widely used in clinical trials investigating the health effects of soy protein and isoflavones (Baum *et al.* 1998; Potter *et al.* 1998; Duncan *et al.* 1999; Alekel *et al.* 2000; Wangen *et al.* 2000; Dent *et al.* 2001; Teede *et al.* 2001; Wangen *et al.* 2001; Jenkins *et al.* 2002; Persky *et al.* 2002; Tonstad *et al.* 2002) is Supro® made by Protein Technologies International. However, the physiological effects of a probiotic soymilk prepared from Supro® SPI on the health status of postmenopausal women have not been studied previously.

2.1.3 Soy germ

Soy germ (SG) or hypocotyl is removed from soybeans by mechanical cracking of the soybean hull and separating from the cotyledons. SG is only a small component of the soybean (2% w/w) and is very compact; hence, it is difficult to separate from other soybean parts commercially, requiring up to 180 kg of soybeans to manufacture 0.5 kg of SG (Schryver, 2002). Despite the difficulties associated with its preparation, SG is still widely used as a soy ingredient due to its nutritional content (Liu, 1997; Schryver, 2002; Uzzan & Labuza, 2004). Apart from containing a considerably higher concentration of isoflavones (Kudou *et al.* 1991), SG also contains significantly higher amounts of saponins, phytosterols, linoleic and linolenic acid, and vitamin E on a per gram basis compared to soy protein concentrate, soy flour and SPI (Schryver, 2002). With respect to macronutrient composition, SG comprises approximately 38 to 40% (w/w) protein, 16% (w/w) fat (predominantly polyunsaturated), 25% (w/w) carbohydrate and 3% (w/w) fibre (Schryver, 2002).

In recent times, a SG product named IsoLife® has been the focus of a number of studies investigating isoflavone bioavailability (Hendrich *et al.* 2001; Zhang *et al.* 2001; Richelle *et al.* 2002) (discussed in section 2.5.7 and in Chapter 7.0), oestrogenic activity of glycitein (Song *et al.* 1999), prebiotic effects (De Boever *et al.* 2000), and the bone health of postmenopausal women (Chen *et al.* 2003) (see section 2.5.13). In a study by Song *et al.* (1999), glycitein was isolated from IsoLife® SG, as it was discovered in the previous year that this soy ingredient was a rich source of glycitein and its glucosidic isomers (Song *et al.* 1998), and its biological activity was assessed in comparison to daidzein, genistein and 17β-estradiol. Results indicated that glycitein had weak oestrogenic activity comparable to that of daidzein and genistein but much lower than that of 17β-estradiol (Song *et al.* 1999). De Boever *et al.* (2000) investigated the prebiotic effects of IsoLife® SG in an *in vitro* simulator of the human intestinal microbial system and found that after two weeks of adding 2.5 g SG powder per day there was a significant increase in the population of *Lactobacillus* sp. Furthermore, the concentration of short chain fatty acids increased by ~30% due mainly to a significant increase in acetic and propionic acids (De Boever *et al.* 2000). The prebiotic effects of SG are most likely due to its high concentration of oligosaccharides and fibre (total of

approximately 15 g per 100 g) (Schryver, 2002). Due to its potential health benefits, SG is used in a variety of health foods including breakfast cereals, whole-grain breads, muesli, nutrition bars, and granola. Finer particle sizes of SG are also included into protein beverages and smoothies (Schryver, 2002). However, there are no isoflavone aglycone-enriched, probiotic soymilk products made from SG powder currently on the Australian marketplace.

2.1.4 Soymilk

Traditional soymilk is the aqueous extract of whole soybeans and is thought to have originated in China during the second century BC. Today, soymilk is consumed by many Asian cultures in much the same way dairy milk is consumed in the West. Soymilk resembles dairy milk in appearance and composition. In comparison to dairy milk, traditional soymilk contains higher levels of protein (~3.6 g per 100 g), less fat (~2.0 g per 100 g), no cholesterol, lower levels of carbohydrate (~2.9 g per 100 g), a rich source of phytoestrogens, but only one-sixth of the calcium content of dairy milk (~15 mg per 100 g soymilk) (Liu, 1997). Despite some of its nutritional advantages, soymilk is not popular amongst Western cultures due to its aroma, flavour profile and aftertaste, frequently described as 'beany' or 'bitter'. In contrast, Asian populations are accustomed to the taste of soymilk through centuries of consumption (Liu, 1997).

Soymilk prepared by traditional Chinese and Japanese methods is generally associated with the greatest amount of 'beaniness' and 'bitterness', but is still the most widely consumed amongst Asian people because of its simple preparation. Basically, whole soybeans are soaked in water overnight, then washed and ground with fresh water at a water to bean ratio between 8:1 and 10:1, and then filtered through a cloth. The residue, known as okara, is separated and the filtrate is boiled for a few minutes before serving (Liu, 1997). However, knowledge on the chemistry of 'beany' flavour formation from numerous studies conducted in the 1960s and 1970s has changed soymilk preparation using whole soybeans. Wilkens *et al.* (1967) and Wilkens & Lin (1970) reported that the characteristic flavour of soymilk was mainly the result of the peroxidation of polyunsaturated fatty acids or esters catalysed by the enzyme lipoxygenases (discussed in section 2.3). During soymilk preparation, the grinding of the soybeans in the presence of water is suggested to cause the

objectionable 'beany' flavour, as lipoxygenases and the lipid fraction are liberated from the cotyledons and allowed to react (Nelson *et al.* 1971). Wilkens *et al.* (1967) reported that the temperature of the bean-water mixture had a great effect on the formation of volatiles during grinding, with temperatures of 80°C and above inactivating lipoxygenases and minimising the peroxidation of lipids. Consequently, new methods of soymilk preparation using either whole or dehulled soybeans were developed to improve soymilk flavour, including the Cornell (Wilkens *et al.* 1967), Illinois (Nelson *et al.* 1976) and rapid hydration hydrothermal cooking (RHHC) (Johnson *et al.* 1981). According to Golbitz (1995), the Cornell method produces a soymilk with an improved beany flavour, and the soymilk made by the Illinois and RHHC methods had no beany flavour. Nevertheless, Shurtleff & Aoyagi (1984) developed a far simpler method of preparing soymilk with no beany flavour by reconstituting protein-rich, defatted soy ingredients, like SPI.

2.2 Soybean oligosaccharides

Mature soybeans contain soluble oligosaccharides raffinose [β-D-fructofuranosyl-*O*-α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside] and stachyose [β-D-fructofuranosyl-*O*-α-D-galactopyranosyl-(1→6)-α-D-galactopyranoside-(1→6)-α-D-glucopyranoside], at concentrations of 0.1 to 0.9% (w/w) and 1.4 to 4.1% (w/w) of dry mass, respectively (Hymowitz *et al.* 1972). Raffinose and stachyose consist of sucrose linked (via α-galactoside bonds) to 1 or 2 galactose moieties, respectively (Figure 2.2). Among the soluble carbohydrates found in soybeans (e.g. glucose and sucrose), raffinose and stachyose receive more attention because their presence is linked to the flatulence and abdominal discomfort associated with soybean and soy food consumption, and are often referred to as anti-nutritional factors (Liu, 1997). The small intestine of humans and animals does not synthesise α-galactosidase necessary for hydrolysing the α-galactosidic linkage present in raffinose and stachyose. Consequently, almost all of the ingested raffinose and stachyose reaches the colon. Colonic bacteria produce a number of saccharolytic enzymes, including α-galactosidase, necessary to ferment these sugars, and copious quantities of carbon dioxide, hydrogen, nitrogen and methane are released during the fermentation process

depending on the individual's diet and intestinal microbial composition (Steggerda *et al.* 1966; Steggerda & Dimmick, 1966; Cristofaro *et al.* 1974; Suarez *et al.* 1997; Suarez *et al.* 1999).

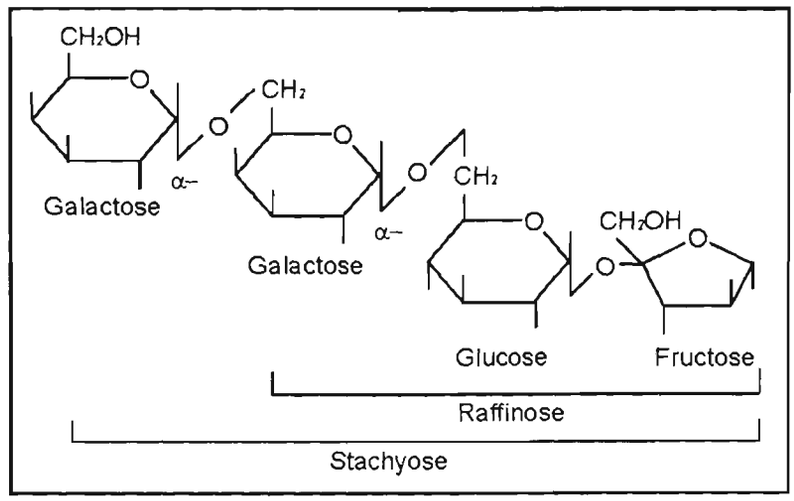

Figure 2.2 Chemical structures of raffinose and stachyose. Adapted from Liu (1997).

The presence of raffinose and stachyose in soybeans is suggested to be the result of the metabolic pathway shown in Figure 2.3. Galactinol (1-O-α-galactopyranosyl-D-myo-inositol), the product of the metabolic pathway involving glucose-6-phospate, myo-inositol and uridine diphosphate galactose, serves as a galactose donor for reactions in which sucrose is converted to raffinose and raffinose to stachyose catalysed by galactinol-sucrose galactosyltransferase (raffinose synthase) and galactinol-raffinose galactosyltransferase (stachyose synthase), respectively (Suarez *et al.* 1999).

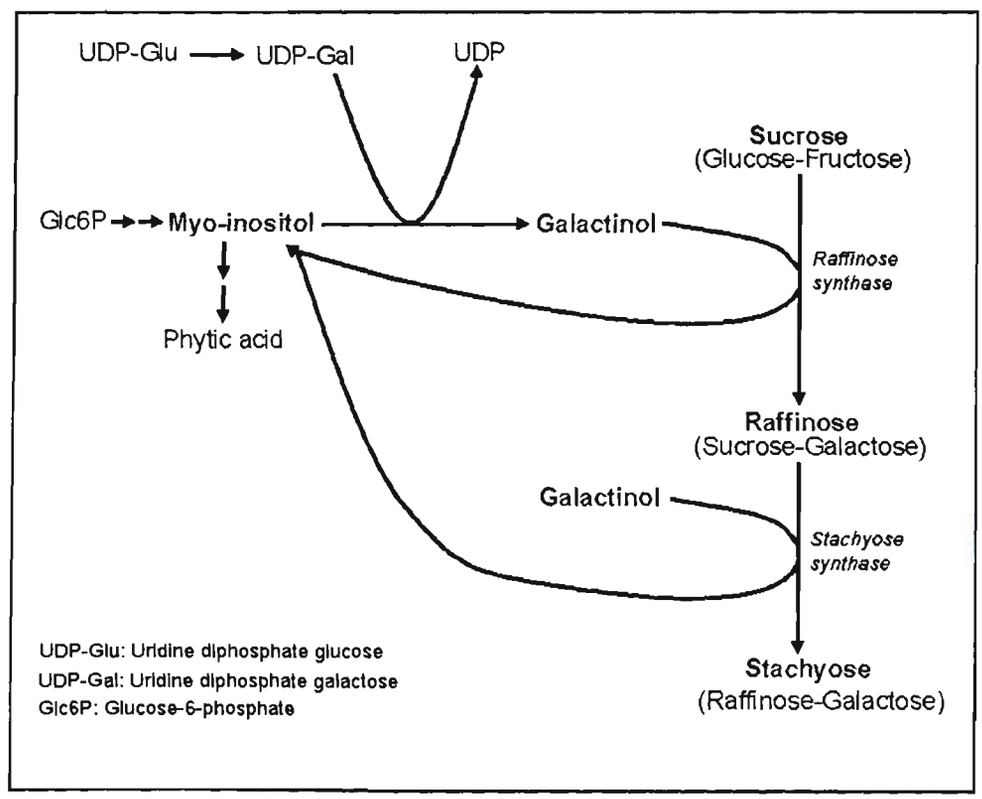

Figure 2.3 Metabolic pathways for the synthesis of raffinose and stachyose from sucrose in soybeans. Adapted from Suarez *et al.* (1999).

Unlike other anti-nutritional factors in soybeans (e.g. trypsin inhibitors and lectins), oligosaccharides are heat stable and heat treatment alone is ineffective in eliminating them during soy food manufacture (Liu, 1997). As a result, alternative approaches to reducing oligosaccharides in soy-based foods have been developed. In the production of soy protein concentrates, Rackis *et al.* (1970) developed an effective method of aqueous alcohol extraction to reduce the content of oligosaccharides. Methods of enzymatic hydrolysis of oligosaccharides have also been developed using either endogenous saccharolytic enzymes produced during germination (Abdullah *et al.* 1984; Guimarães *et al.* 2001) or exogenous enzymes produced by mold (Calloway *et al.* 1971), lactic acid bacteria (Mittal & Steinkraus, 1975; Pinthong *et al.* 1980) and *Bacillus subtilis* (Sarkar *et al.* 1997) in the production of fermented soy foods, or industrial preparations of microbial α-galactosidase to treat soymilk (Cruz *et al.* 1982). Species of *Bifidobacterium* have also exhibited an ability to hydrolyse raffinose and stachyose during the fermentation of whole bean soymilk, reducing the concentration of oligosaccharides to very low or non-detectable levels (Scalabrini *et al.* 1998; Hou *et al.* 2000; Desai *et al.* 2002; Wang *et al.* 2003). Most strains of bifidobacteria produce α-galactosidase needed to hydrolyse these sugars (Desjardins & Roy, 1990; Scalabrini *et al.* 1998).

Although the presence of oligosaccharides in soybeans and soy foods is generally considered undesirable as they cause flatulence and abdominal discomfort, clinical studies have also shown that the ingestion of oligosaccharides benefit the health of humans by acting as 'prebiotics'. Studies by Benno *et al.* (1987), Masai *et al.* (1987) and Hayakawa *et al.* (1990) reported that the ingestion of oligosaccharides (raffinose and/or stachyose) increased the populations of indigenous bifidobacteria in the colon which, by their antagonistic effects, suppressed the growth activity of putrefactive bacteria, according to bacterial populations in faecal specimens. Hence, the potential advantages to human health associated with prebiotic oligosaccharides, because of their ability to adjust intestinal microbial balance, include reducing toxic metabolites and detrimental enzymes (anti-carcinogenic effects), preventing diarrhoea (due to the reduction of pathogenic bacteria), preventing constipation (due to the production of high levels of short chain fatty acids by bifidobacteria), and enhancing the production of vitamins due to increased activity of bifidobacteria (Crittenden, 1999).

2.3 Aldehydes in soy foods

The objectionable odour associated with various soy foods is predominantly due to the presence of volatile carbonyl compounds, including aldehydes, ketones and alcohols (Liu, 1997). Among the volatile compounds, the aldehyde hexanal is primarily responsible for the 'beany' flavour of soy foods due to its extremely low flavour threshold (< 1 ppm) (Fujimaki *et al.* 1965). The formation of these volatile compounds is the result of the chemical degradation of the lipid fraction of the soybean catalysed by an enzyme naturally found in the soybean's cotyledons known as lipoxygenases (linoleate oxidoreductase). Soybean seeds are the richest known source of lipoxygenases, of which four have been isolated and identified as L-1, L-2, L-3a and L-3b (Liu, 1997). In the formation of off-flavours, lipoxygenases catalyse hydroperoxidation of linoleic acid and other polyunsaturated lipids that contain a *cis,cis*-1,4-pentadiene moiety, in the presence of molecular oxygen. The primary products of this reaction are hydroperoxides (Liu, 1997). Robinson *et al.* (1995) reported that there are three steps assumed for the reaction: (1) activation of the native enzyme (as described in section 2.1.4 with regard to soymilk preparation); (2) removal of a proton from the activated methylene group; and (3) insertion of the oxygen into the substrate molecule. Hydroperoxides are then degraded into a variety of carbonyl compounds including hexanal and pentanal (Figure 2.4) by hydroperoxide lyases and/or isomerases (Liu, 1997).

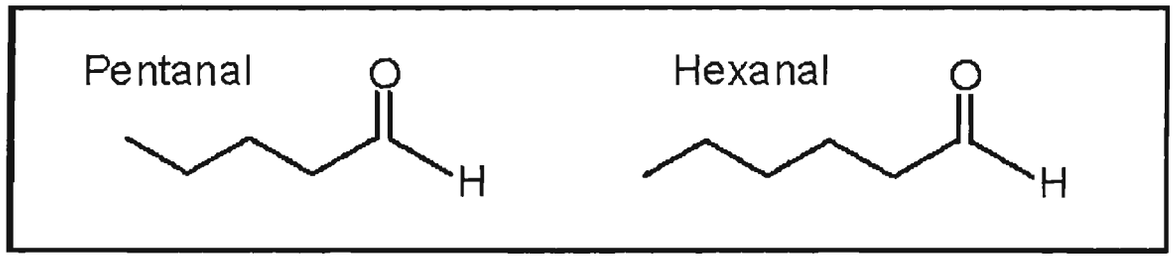

Figure 2.4 Chemical structures of aldehydes pentanal and hexanal.

Most non-fermented soy foods contain low quantities of aldehydes (more so hexanal than pentanal), including soymilk (Wilkens *et al.* 1967; Nelson *et al.* 1976; Scalabrini *et al.* 1998; Desai *et al.* 2002), defatted and full fat soybean flour, soy protein concentrates (Arai *et al.* 1970; Maheshwari *et al.* 1997), SPI (Inouye *et al.* 2002), and soybean oils (List *et al.* 1977). In the production of traditional soymilk, heat treatment is generally used to inactivate lipoxygenases and prevent the

formation of objectionable aldehydes, as described in section 2.1.4. However, in the preparation of soy proteins, using heat sufficient to inactivate lipoxygenases often leads to some insolubilisation of proteins, loss of protein functionality, and introduction of a cooked or toasted flavour. For this reason, several techniques have been developed using a combination of steps including a milder heat treatment (e.g. 45°C), adjustment of the moisture content of soybeans (between 16 and 18% w/w), pH adjustment (in either acidic or alkaline conditions as lipoxygenases is most stable at pH 6.0) and/or soaking the soybeans in 29 to 90% aqueous alcohol (Baker & Mustakas, 1973; Elridge *et al.* 1977; Borhan & Snyder, 1979; Brown *et al.* 1982). Other researchers have developed methods to eliminate aldehydes from soy proteins after they have formed using numerous techniques of deodorisation, including enzymes such as aldehyde dehydrogenase (Takahashi *et al.* 1980; Matoba *et al.* 1985) and porcine liver aldehyde oxidase (Maheshwari *et al.* 1997), microorganisms (Kobayashi *et al.* 1992), ultracritical carbonic dioxide gas (Maheshwari *et al.* 1995), and solid adsorbents made of polystyrene, polymethacrylate, zeolite and charcoal (Inouye *et al.* 2002). With regard to commercial whole bean soymilks, Scalabrini *et al.* (1998) and Desai *et al.* (2002) found that some strains of *Bifidobacterium* completely metabolised hexanal and pentanal during fermentation; hence, reducing the objectionable 'beaniness' of these soymilks. However, previous studies have not analysed the concentrations of hexanal and pentanal in soymilk made from SPI and whether or not these volatiles can be completely eliminated by bifidobacteria during fermentation (see Chapter 5.0). Inouye *et al.* (2002) stated that the offensive odour associated with SPIs, predominantly due to the presence of hexanal, appears to limit their utilisation as a soy ingredient and protein source for humans.

2.4 Phytoestrogens

Phytoestrogens, *phyto-* derived from the Greek word *φυτό* meaning plant, are a class of compounds found in plants that are structurally and functionally similar to mammalian oestrogen. In plants, phytoestrogens are synthesised from phenylpropanoids and simple phenols (Hahlbrock, 1981) and function primarily as antioxidants (Albertazzi & Purdie, 2002). In humans, phytoestrogens exert oestrogen-like actions because of their structural homology to 17β-estradiol (Knight & Eden, 1996).

The principal human oestrogen, 17β-estradiol, possesses greater oestrogenic activity than endogenous oestrogens estriol and estrone (Gruber *et al.* 2002) (Figure 2.5). Oestrogens influence the growth and functioning of female and male reproductive tissues, maintain the skeletal and central nervous system, and protect against cardiovascular disease, some cancers and aging skin (Gruber *et al.* 2002; Ruggiero & Likis, 2002).

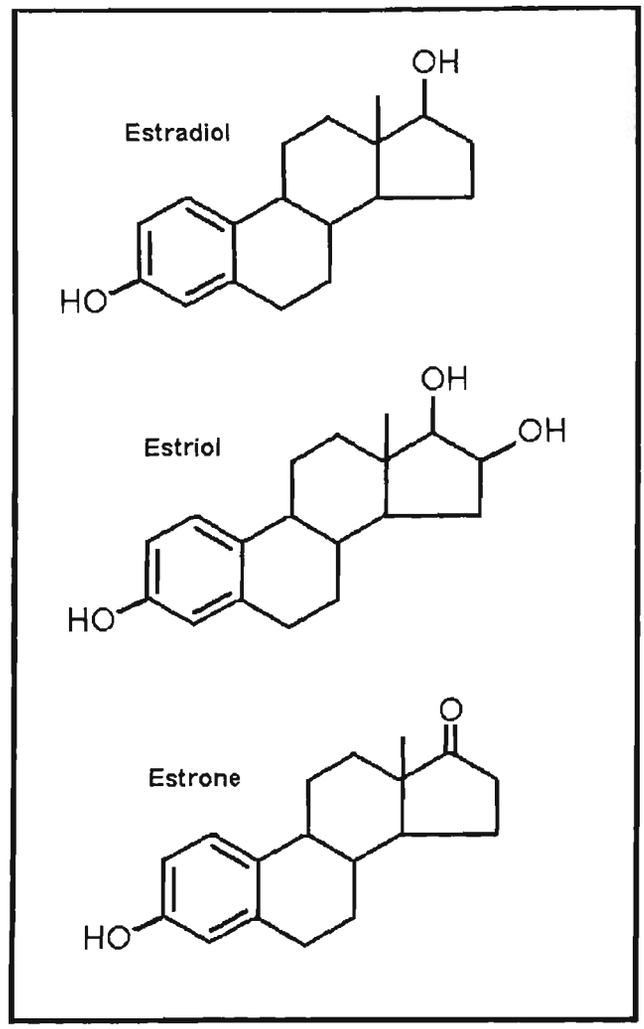

Figure 2.5 Chemical structures of human endogenous oestrogens. Adapted from Ososki & Kennelly (2003).

Phytoestrogens are unique in that they can function oestrogenically as oestrogen agonists and anti-oestrogenically as antagonists. As oestrogen agonists, phytoestrogens mimic endogenous oestrogens and cause oestrogenic effects. Alternatively, as oestrogen antagonists they may block or alter oestrogen receptors (ERs) and prevent oestrogenic activity. Consequently, phytoestrogens have been classified as selective oestrogen receptor modulators (SERMs) (Brzezinski & Debi, 1999).

SERMs are non-steroidal chemicals with a similar structure to 17β-estradiol and an affinity toward ERs (Riggs & Hartmann, 2003), but function as agonists and antagonists depending on the tissue, ER, and concentration of circulating endogenous oestrogens (Gruber *et al.* 2002). ERs bind with steroidal as well as numerous non-steroidal compounds (Ososki & Kennelly, 2003) comprising an aromatic ring and hydroxyl group (phenol), which is important for binding effectiveness (Anstead *et al.* 1997). Phytoestrogens bind to two types of ERs: oestrogen receptor α (ERα) and β (ERβ). Mosselman *et al.* (1996) discovered that phytoestrogens have a higher binding affinity for ERβ, which is distributed in human tissues such as bone, brain, vascular endothelia and bladder (Setchell, 1998).

2.4.1 Classification and sources

Of the several classes of phytoestrogens, the most ubiquitous are the phenolic oestrogens, including isoflavones, coumestans and lignans (Ososki & Kennelly, 2003). However, isoflavones have been the main focus of research. Investigation of the oestrogenic activity of isoflavones originally resulted from the recognition of 'clover disease' (reproductive disorder) in Australian sheep (Bennetts *et al.* 1946), whose diet was predominantly clover (*Trifolium subterraneum* L.). Isoflavones are primarily found in the Fabaceae botanical subfamily (Leguminosae family), including soybean (*Glycine max* L. Merr.), alfalfa (*Medicago sativa* L.), bean (*Phaseolus vulgaris* L.), and red clover (*Trifolium pratense* L.). Like isoflavones, coumestans are also found in legumes, particularly in sprouts of alfalfa, mung bean (*Vigna radiata* L., Fabaceae) (Lookhart, 1980; Mazur, 1998) and soybean (Ibaretta *et al.* 2001), and are especially high in clover (Franke *et al.* 1995). The main coumestans which exert oestrogenic effects are coumestrol and 4′-methoxycoumestrol (Ososki & Kennelly, 2003) (Figure 2.6). Lignans, on the other hand, are commonly found in flaxseed (*Linum usitatissimum* L., Linaceae) (Kurzer & Xu, 1997), rye (*Secale cereale* L., Poaceae) (Thompson *et al.* 1991), grains and cereals (Mazur, 1998), and brewed green and black tea and coffee (Mazur *et al.* 1998). Secoisolariciresinol and matairesinol are the most studied lignans, which are converted by intestinal bacteria into oestrogenically active enterodiol and enterolactone, respectively (Setchell & Adlercreutz, 1988) (Figure 2.7).

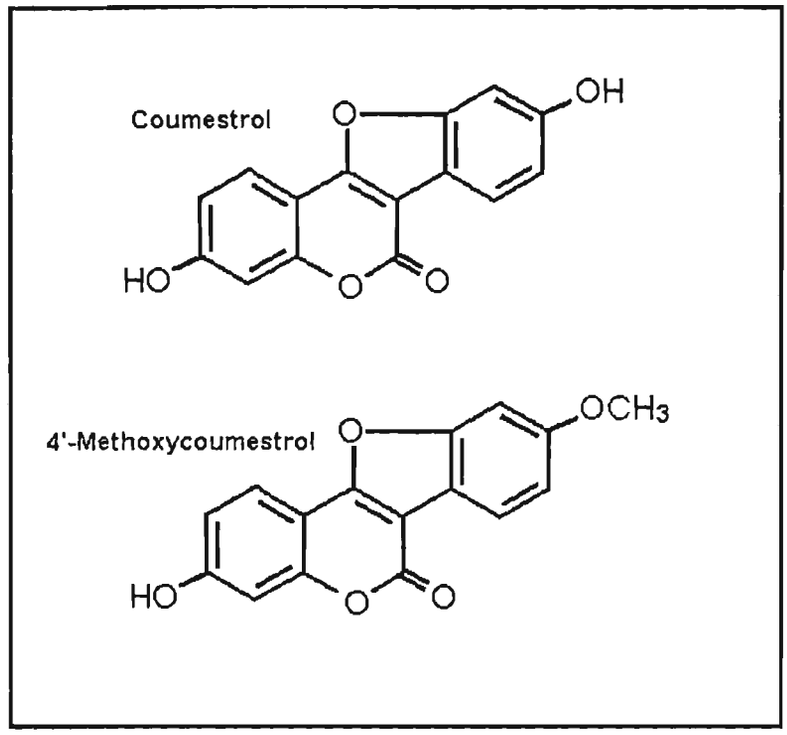

Figure 2.6 Chemical structures of coumestans. Adapted from Ososki & Kennelly (2003).

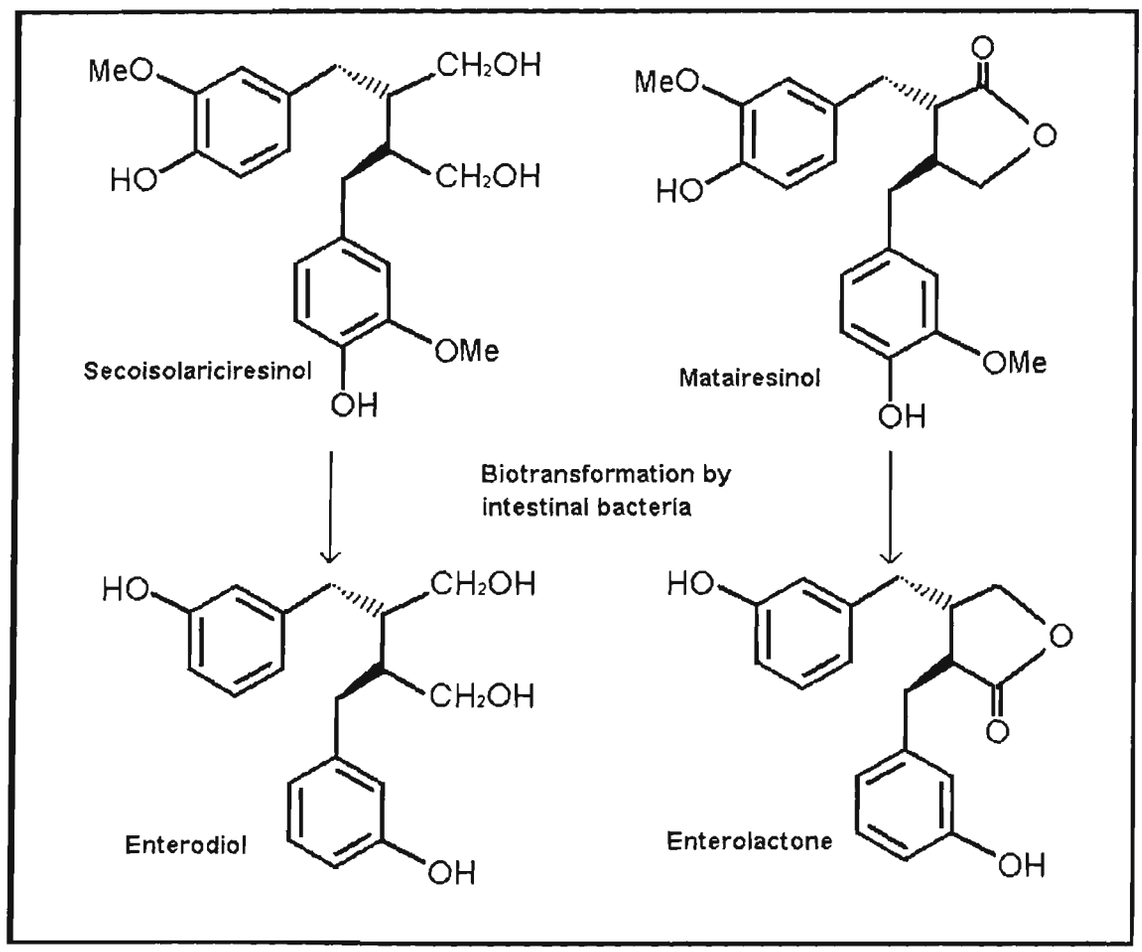

Figure 2.7 Chemical structures of mammalian lignans, enterodiol and enterolactone, and the configuration of their plant-derived precursors. Adapted from Ososki & Kennelly (2003).

2.5 Isoflavones

2.5.1 Chemistry

Isoflavones belong to a group of compounds known as flavonoids, which include the largest and most diverse range of plant phenolics. Isoflavones and other subclasses of flavonoids, including flavones, flavonols, flavanols, aurones, chalcones and anthocyanin pigments, share a basic structure consisting of two benzyl rings joined by a three-carbon bridge, which may or may not be closed in a pyran ring (Deshphande *et al.* 1984). The basic isoflavone structure is shown in Figure 2.8 and differs from flavone in that the benzyl ring B is joined at position 3 instead of position 2. Isoflavones are present in just a few botanical families, including Fabaceae, Asteraceae, Juglandaceae, Iridaceae, and Euphorbiaceae (Dewick, 1993; Mazur, 1998), because of the limited distribution of the enzyme chalcone isomerase, which converts 2(*R*)-naringinen (a flavone precursor) into 2-hydroxydaidzein (Coward *et al.* 1993). In the human diet, the most abundant source of isoflavone is from soybeans.

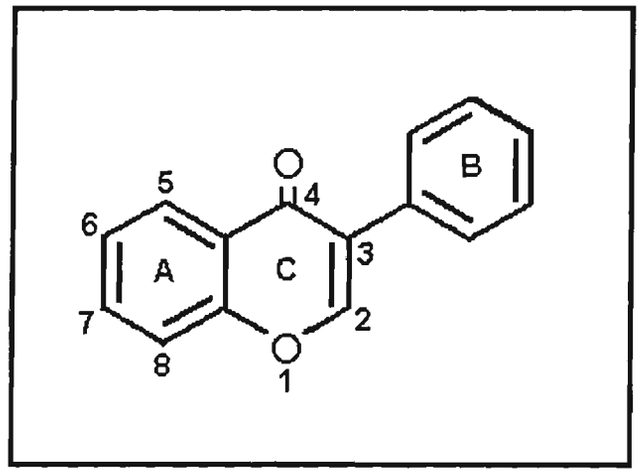

Figure 2.8 Basic chemical structure of isoflavone. Adapted from Liu (1997) and Ososki & Kennelly (2003).

Soybeans and soy foods contain twelve isoflavone isomers. The isomers with an oestrogen-like configuration are the aglycone forms of daidzein (4′,7-dihydroxyisoflavone), genistein (4′,5,7-trihydroxyisoflavone) and glycitein (4′,7-dihydroxy-6-methoxyisoflavone) (Figure 2.9). There are also nine conjugated isoflavone isomers that have an attached glucose moiety at position 7 of the A-ring. The simple glucosidic isoflavone isomers are the β-glucosides (daidzin, genistin and glycitin) which can be further bound to either an acetyl (6″-*O*-acetylglucosides) or malonyl (6″-*O*-

malonylglucosides) group (Figure 2.10). Formononetin and biochanin-A are also found in soy and are 4′-methyl ethers of daidzein and genistein, respectively (Figure 2.11). Ibaretta *et al.* (2001) found high levels of formononetin and biochanin-A in soy seeds, both at approximately 0.7 mg per gram dry weight. However, formononetin and biochanin-A are generally classified as clover-derived isoflavones which are demethylated in the mammalian gastrointestinal tract into their respective aglycone isomers (Heinonen *et al.* 2002).

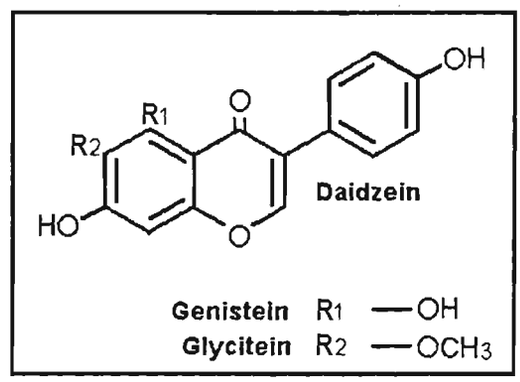

Figure 2.9 Chemical structures of isoflavone aglycone isomers. Adapted from King & Bignell (2000).

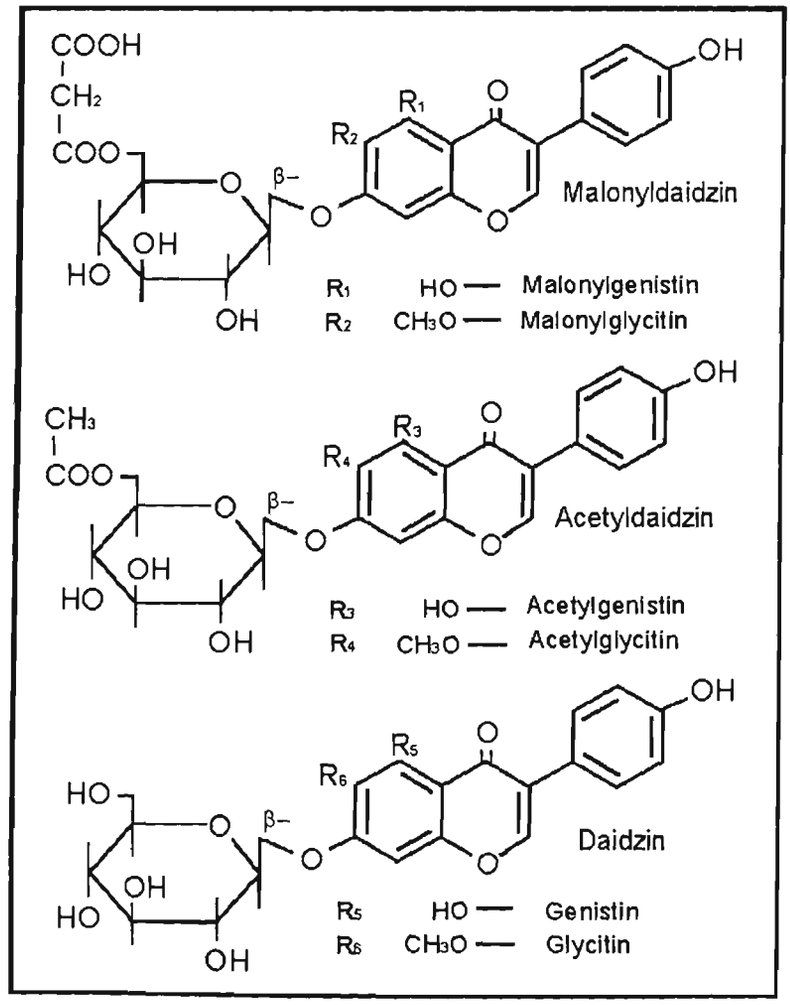

Figure 2.10 Chemical structures of glucosidic isoflavone isomers. Adapted from King & Bignell (2000).

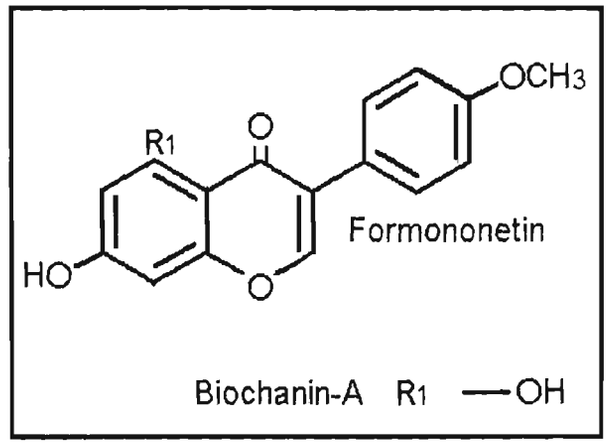

Figure 2.11 Chemical structures of methoxylated isoflavone isomers found predominantly in clover. Adapted from Ososki & Kennelly (2003).

2.5.2 Isoflavone variation in soybeans

Wang & Murphy (1994) and Tsukamoto *et al.* (1995) reported that the content and composition of isoflavones in soybeans varied considerably between species and is also influenced by agricultural factors such as crop year, growth location and climate. Wang & Murphy (1994) found that the isoflavone content of eight American soybean varieties ranged between 1.2 and 4.4 mg per gram, with three Japanese varieties comprising a lower concentration range of 1.2 to 2.3 mg isoflavone per gram. The composition of isoflavone isomers also varied considerably between varieties of soybean, with Japanese soybeans containing higher levels of malonylglycitin and higher ratios of malonyldaidzin to daidzin and malonylgenistin to genistin. In a more recent study, King & Bignell (2000) analysed the concentration of isoflavones in four soybean varieties grown in Queensland, Australia, and found that the total isoflavone level ranged from 0.51 to 1.7 mg per gram (expressed on the basis of aglycone constituents). For the four cultivars, planted in both January and December 1998, the mean isoflavone content of cultivars planted in December was approximately 20% higher than the mean for the same cultivars planted in January ($P<0.001$). Of the isoflavone isomers, genistein and its glucosides were consistently found at higher levels in all cultivars, with glycitein and its glucosides consistently found at lowest concentration (King & Bignell, 2000). Considering the large amount of variation in isoflavone composition between soybeans, even of the same variety, it is expected that this will cause considerable variation in the isoflavone composition of

soy-based foods and ingredients, not to mention the additional effects of food processing on isoflavone variation.

2.5.3 Composition of isoflavones in SPI and SG

In a recent study, Setchell & Cole (2003) quantified isoflavones in thirty separate samples of two types of SPI purchased over a 3-year period and reported that total isoflavone content (expressed on the basis of aglycone constituents) varied between 0.7 and 1.5 mg per gram for one type and between 0.3 and 1.1 mg per gram for the other. Of the individual isomers, β-glucosides of genistein and daidzein were consistently found at highest concentration, with malonylglucosides found at extremely low levels (Setchell & Cole, 2003). SPIs analysed by Setchell & Cole (2003) were used in the manufacture of soy foods (e.g. soymilk) by Sanitarium Health Food Company (Cooranbong, NSW, Australia) over this 3-year period (Setchell & Cole, 2003). Hence, the isoflavone content of soy foods produced by Sanitarium most likely reflect the considerable variation in isoflavone in the SPI, in addition to other steps along the production process which may cause changes in isoflavone composition.

To investigate the effects of the SPI manufacturing process on isoflavone composition, Wang & Murphy (1996) conducted a mass balance study of isoflavones at each processing step between the raw material (defatted soybean flour) and protein isolate and discovered that there were significant losses of 53% of total isoflavone contents ($P<0.05$). The alkaline extraction step caused the major losses of isoflavones into the alkaline-insoluble fraction, which is typically discarded (Wang & Murphy, 1996). In addition, alkaline extraction changed the composition of isomers, with the concentration of aglycones (genistein, daidzein and glycitein) increasing in the soluble portion compared to the dry starting material (Wang & Murphy, 1996). Consequently, the final product contained a higher percentage of aglycone compared to the defatted soy flour. However, Wang & Murphy (1996) did not spray-dry their protein isolate, typically carried out in the commercial production of SPI, so as to observe the effects of heat processing. Studies by Barnes *et al.* (1994) and Coward *et al.* (1998) reported that thermal processing increases the concentration of acetylglucosides in soy ingredients and soy foods as a result of the decarboxylation of

malonylglucosides. In some cases deacetylation occurred, increasing the concentration of β-glucosides (Barnes *et al.* 1994; Coward *et al.* 1998). Nevertheless, even though Barnes *et al.* (1994) and Coward *et al.* (1998) noted intra-conversions between isoflavone isomers, they observed very little change in total isoflavone levels.

In comparison to the relatively low levels of isoflavone expected in SPI (generally 0.5 to 2 mg per gram), SG powder is considered the richest source of isoflavone amongst the commonly used soy ingredients (Schryver, 2002). SG contains 20 to 23 mg isoflavone per gram, with only 2 to 4% in a bioactive aglycone configuration (Song *et al.* 1998; Setchell *et al.* 2001). Of the isoflavone isomers, Song *et al.* (1998) reported that daidzein and its glucosidic isomers were found at highest concentration in SG powder followed by glycitein and genistein and their respective isomers, with the acetylglucosides being the predominant structures. This may be due to decarboxylation of malonylglucosides caused by roasting, a processing step which is commonly used in the preparation of SG powder.

2.5.4 Composition of isoflavones in soymilk

Due to increasing consumer awareness of the presence of bioactive, health-promoting isoflavones in soy foods, numerous studies have analysed the concentrations of isoflavones in some of the most popular commercial soy products (including soymilk) available in food marketplaces around the world. With respect to soymilk, products available in the US (Barnes *et al.* 1994; Coward *et al.* 1998; Murphy *et al.* 1999), UK (Wiseman *et al.* 2002), Singapore, and Hawaii (Franke *et al.* 1999) have been analysed for their isoflavone levels. On the whole, the total isoflavone levels in soymilks (all types) available in these countries ranged between 3.5 and 17.3 mg per 100 g (sum of genistein, daidzein and glycitein and their respective glucosidic isomers).

With regard to Australian soymilk products, King & Bignell (2000), Hutabarat *et al.* (2001) and Setchell & Cole (2003) also found considerable variation in isoflavone composition between different brands of soymilk. King & Bignell (2000) analysed the isoflavone composition of five brands of soymilk purchased in Adelaide (South Australia), without disclosing the brand names,

and found that the total isoflavone content varied between 5.8 and 23.7 mg aglycone equivalents per 100 g. The predominant isoflavone structures found in each of these soymilk brands were the β-glucosides of daidzein, genistein and glycitein, with the acetylglucosides not detected in any of the soymilk samples (King & Bignell, 2000). However, King & Bignell (2000) did not provide information about the types of soymilk that were analysed, that is, whether they were made from soy protein or whole bean. Hence, correlations between the type of soymilk and its influence on isoflavone composition could not be made. In a later study, Hutabarat *et al.* (2001) provided both the product name and type of raw material used in soymilk manufacture, but in this case the authors did not analyse for glycitein and its glucosidic isomers in each of the soymilk samples. Instead, the total concentrations of genistein and daidzein and their respective glucosides were analysed in five commercially available whole soybean soymilks [Vitalife (Natural Foods), Vitasoy (Vitasoy International), Natures (Pureharvest), So Natural (Natural Foods) and Soya drink (Soya King)] and SPI-based soymilks [So Good and So Good Lite (Sanitarium), Good Life (Berrivale Orchards), Soy Drink (No Frills) and Soy Drink (Sun Gold)]. Sun Gold Soy Drink (7.8 mg isoflavone per gram) and So Natural (9.5 mg isoflavone per gram) contained the highest total isoflavone content of the SPI and whole bean soymilks, respectively. It was found that whole soybean soymilks contained a higher isoflavone concentration than SPI-based soymilks, with a mean concentration of 7.3 and 5.8 mg isoflavone per gram, respectively (Hutabarat *et al.* 2001). This is expected considering the lower isoflavone levels in SPI due to losses in protein isolation.

In the most recent study on isoflavones in Australian commercial soymilks (40 varieties), Setchell & Cole (2003) also found that the mean total isoflavone content of soymilks made from SPI, at 3.0 ± 0.6 mg per 100 mL, was significantly lower than the mean isoflavone content of whole soybean soymilks ($P<0.0001$), which averaged 6.4 ± 2.2 mg per 100 mL. However, there was greater variation in total isoflavone concentration amongst whole bean soymilk brands, with values varying up to 5-fold. With respect to shelf life, Setchell & Cole (2003) found that there was no trend toward lower total isoflavone concentrations with the aging of ultra high temperature treated (UHT) whole bean and SPI-based soy milks within the range of 61 to 359 days of storage. Nevertheless, the intense heating step used in commercial production caused the malonyl conjugates to be at very low

levels (between 0 and 4.9% of total isoflavone), most likely due to decarboxylation, as the acetyl conjugates were much more prominent (up to 40.7% of total isoflavone). The proportion of free aglycone in these soymilks was consistently found at low levels, between 0.8 and 17.5% of total isoflavone (Setchell & Cole, 2003).

In the scientific literature, the presence of isoflavones in soy foods as bioactive, health-promoting compounds generally overshadows their adverse effects on the flavour profile of soy foods. Even though the lipid fraction and formation of aldehydes is typically associated with the undesirable 'beaniness' of soy foods, isoflavones are thought to be responsible for bitterness and astringency (Huang *et al.* 1981). Chang *et al.* (1990) stated that genistin was a bitter and astringent isoflavone glucoside, but Murphy *et al.* (1998) suggested that the aglycones, genistein and daidzein, are responsible for the objectionable taste of traditional soy foods made from soymilk. Shurtleff & Aoyagi (1984) reported that soymilk made from SPI had a bland taste with no 'beaniness' due to the reduced lipid fraction and low concentration of isoflavone. However, a soymilk of low isoflavone content may be of less health benefit compared to a bitter, isoflavone aglycone-rich soymilk.

2.5.5 Enzymic hydrolysis of isoflavones in soy foods

The hydrolysis of isoflavone glucosides by saccharolytic enzymes has been well documented in the scientific literature, during the preparation of traditional soymilk (Matsuura *et al.* 1989; Matsuura & Obata, 1993) and soy food (Wang & Murphy, 1996), and in liquid mediums containing isoflavone-rich soy ingredients (Pandjaitan *et al.* 2000; De Boever *et al.* 2001; Choi *et al.* 2002; Xie *et al.* 2003). In an early study, Matsuura *et al.* (1989) reported that daidzein and genistein increased during the soaking of soybeans, the first step of traditional soymilk manufacture, with the maximum production of these isoflavones occurring at 50°C and at pH 6.0. Considering the structure of isoflavone glucosides and the isolation of β-glucosidase from soybeans (Hösell & Todenhagen, 1980), Matsuura *et al.* (1989) concluded that β-glucosidases were involved in the hydrolysis of isoflavone glucosides, causing the increase in aglycone forms. This was supported by the maximum liberation of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside (a specific substrate for β-D-

glucosidases) occurring in soaking water at 50°C and pH 6.0 (Matsuura *et al.* 1989). In a later study, Matsuura & Obata (1993) discovered that incubating β-glucosidases (isolated from soybean cotyledon) in soymilk for 3 h at 45°C caused the hydrolysis of daidzin and genistin, subsequently increasing the concentration of daidzein and genistein. More recently, Pandjaitan *et al.* (2000) and Xie *et al.* (2003) used β-glucosidase to enhance the concentration of bioactive aglycones in soy ingredients (e.g. soy protein concentrate, soy meal and SG powder) via the hydrolysis of isoflavone glucosides.

To observe the effects of fungal enzymes on isoflavone hydrolysis, Wang & Murphy (1996) quantified isoflavones before and after the fermentation of cooked soybeans in the preparation of tempeh (traditional soy food from Indonesia) and found that *Rhizopus oligosporus* hydrolysed daidzin and genistin during growth, increasing the concentration of their respective aglycone forms ($P<0.05$). Studies by De Boever *et al.* (2001) and Choi *et al.* (2002) investigated the effects of saccharolytic enzyme-producing *Lactobacillus* sp. on isoflavone hydrolysis, but reported contrasting conclusions concerning the enzymes involved. Choi *et al.* (2002) grew *Lactobacillus delbrueckii* subsp. *delbrueckii* in MRS broth and soymilk and found that daidzin and genistin (each at 50 μg per mL) were completely hydrolysed into their respective aglycones within 30 min due to the activity of β-glucosidase, consistent with previous studies (Matsuura & Obata, 1993; Matsuura *et al.* 1995). Other lactic acid bacteria, that did not produce β-glucosidase in enzyme assays, were unable to hydrolyse genistin and daidzin (Choi *et al.* 2002). In contrast, De Boever *et al.* (2001) reported that β-galactosidase completely hydrolysed isoflavone glucosides, releasing their aglycone forms, in semi-skimmed milk (containing SG powder) fermented by *Lactobacillus reuteri* at 37°C (overnight incubation). From enzyme assays, De Boever *et al.* (2001) found that the fermented milk exhibited high β-galactosidase activity, thus, in a separate experiment they incubated β-galactosidase (0.5 g per litre) in a suspension of SG powder and confirmed that this enzyme was able to hydrolyse isoflavone glucosides. Like *Lactobacillus* sp., previous studies have shown that *Bifidobacterium* sp. produce a number of saccharolytic enzymes (Desjardins & Roy, 1990),

including β-glucosidase (Tochikura *et al.* 1986), but their potential ability to hydrolyse isoflavone glucosides into aglycones has not been studied previously (see Chapters 3.0, 4.0 and 6.0).

2.5.6 Biological activity

According to *in vitro* studies, isoflavones possess between 0.01 and 0.0001 of the oestrogenic activity of 17β-estradiol on a molar basis (Mayr *et al.* 1992; Markiewicz *et al.* 1993). Despite their relative weakness, isoflavones are still likely to exert oestrogenic actions *in vivo*, considering that serum isoflavone levels can be up to 10,000 times higher than endogenous oestrogen levels in people who consume soy foods (King, 1998). Of the isoflavone isomers, genistein has the highest binding affinity to the recently discovered ERβ (Whitten & Naftolin, 1998), with almost the same affinity as oestrogens (Kuiper *et al.* 1998). Apart from their oestrogenic activity and interaction with ERs, isoflavones (especially genistein) have shown to exert numerous other biological effects which may account for their possible beneficial roles in the prevention and treatment of diseases as diverse as cancer (Messina *et al.* 1994), osteoporosis (Messina & Messina, 2000) and coronary heart disease (Marsh, 2000). *In vitro*, genistein was found to inhibit the growth of a wide range of both hormone-dependent and independent cancer cells (Messina *et al.* 1994; Constantinou & Huberman, 1995), which was later associated with the ability of genistein to influence signal transduction (Weber *et al.* 1999). Furthermore, genistein inhibits the activity of enzymes (e.g. tyrosine-specific protein kinases) and cellular factors that control the growth of cells (Akiyama *et al.* 1987; Akiyama & Ogawara, 1991; Kim *et al.* 1998). In addition, isoflavones possess antioxidant activity (Ruiz-Larrea *et al.* 1997) and may stimulate the immune system (Wang *et al.* 1997; Zhang *et al.* 1999).

2.5.7 Bioavailability

The ability of isoflavones to prevent and potentially treat chronic diseases depends on their bioavailability, which generally refers to the metabolism, absorption, distribution to target tissues and specific sites of action, and excretion of a bioactive ingredient or drug. With respect to absorption, current knowledge indicates that isoflavones in an aglycone configuration are those isomers which are absorbed through the intestinal epithelium. Setchell *et al.* (2002*a*) reported that the isoflavone β-glucosides genistin and daidzin were not absorbed intact across the enterocyte of

healthy adults, and their bioavailability required the hydrolysis of the sugar moiety by intestinal glucosidases. The bioavailability of isoflavones is typically evaluated in terms of plasma concentrations and/or urinary excretion. The metabolic activity of intestinal bacteria is suggested to influence the extent of isoflavone absorption and the concentrations of isoflavone found in plasma and urine (Hendrich, 2002).

A number of pharmacokinetic studies have investigated the bioavailability of isoflavones when ingested in a bioactive aglycone-rich form and compared it to the bioavailability of the glucosidic isomers (Hutchins *et al.* 1995; Izumi *et al.* 2000; Setchell *et al.* 2001; Richelle *et al.* 2002; Zubik & Meydani, 2003). Hutchins *et al.* (1995) fed men and women fermented soybean pieces (tempeh), comprising a greater proportion of isoflavone aglycone, and found that urinary recovery of isoflavone was significantly greater than when the group consumed isoflavone glucoside-rich cooked soybean pieces ($P<0.05$). Izumi *et al.* (2000) analysed concentrations of isoflavone in plasma after a single dose as well as after 2 and 4 weeks of isoflavone aglycone and isoflavone glucoside intake and found that the plasma concentration of daidzein and genistein was significantly greater in subjects consuming an aglycone-rich supplement ($P<0.05$). In more recent studies, Richelle *et al.* (2002) and Zubik & Meydani (2003) reported that the consumption of an isoflavone aglycone-rich beverage and tablet, respectively, did not enhance the bioavailability of genistein, daidzein or glycitein, with both studies showing similar plasma pharmacokinetics after the ingestion of isoflavone aglycone- and glucoside-rich products. In contrast, Setchell *et al.* (2001) reported that the bioavailability of daidzein and genistein was greater when they were ingested as β-glucosides rather than aglycones according to the area under the curve (AUC) of the plasma appearance and disappearance concentrations. In pharmacokinetic studies by Setchell *et al.* (2001), Richelle *et al.* (2002) and Zubik & Meydani (2003), the bioavailability of isoflavone was examined after a single dose was administered. Whereas, Hutchins *et al.* (1995) and Izumi *et al.* (2000), who found that the apparent bioavailability of isoflavone was greater when ingested as an aglycone, analysed urinary and plasma isoflavone profiles over a period of several days or weeks of isoflavone supplementation.

To date, the majority of pharmacokinetic studies have shown that the urinary recovery of daidzein exceeds that of genistein and glycitein in response to either a single dose or daily consumption of soy foods or isoflavone supplements (Xu *et al.* 1994, 1995; Hutchins *et al.* 1995; Lu *et al.* 1996; King & Bursill, 1998; Lu & Anderson, 1998; Xu *et al.* 2000; Zhang *et al.* 2001; Richelle *et al.* 2002; Setchell *et al.* 2003). Hence, the higher urinary excretion of daidzein reflects a greater bioavailability of this isoflavone. However, investigations on plasma isoflavone kinetics in response to a single dose of soy food or isoflavone supplement have not supported the apparent greater bioavailability of daidzein. Setchell *et al.* (2001) reported that the systemic bioavailability of genistein was much greater than daidzein as measured from the AUC of the plasma appearance and disappearance concentrations. Watanabe *et al.* (1998) and Izumi *et al.* (2000) also found that genistein was absorbed more efficiently than daidzein with higher concentrations in plasma and a longer half-life. King & Bursill (1998) concluded that the bioavailability of daidzein and genistein was similar, with the ratio of the AUC for genistein and daidzein equal to the ratio of the concentrations of these isoflavones in the soy meal ingested. Discrepancies observed in previous pharmacokinetic studies may be attributed to inter-individual variation in intestinal microflora of study groups from different age groups, countries and cultural backgrounds, influencing the intestinal metabolism and absorption of isoflavones.

2.5.8 Isoflavone metabolites

Deglycosylation of isoflavone glucosides has to occur prior to isoflavone absorption (Heinonen *et al.* 2002) and is probably carried out by the hydrolytic actions of both mammalian and microbial glucosidases. For many years it was assumed that only the β-glucosidases of intestinal microflora were responsible for hydrolysing isoflavone glucosides. However, recent studies by Day *et al.* (1998) showed that β-glucosidases of the human small intestine effectively hydrolysed the glucose residue of isoflavone conjugates. Apart from isoflavone glucosides, aglycones also undergo structural changes in the intestinal tract, instigated by complex microbial enzyme systems. A comprehensive study on isoflavone metabolism by Joannou *et al.* (1995) reported that the reductive metabolism of daidzein resulted in the formation of equol (see section 2.5.9) and *O*-desmethylangolensin (Figure 2.12). Furthermore, reductive metabolism transformed genistein into

dihydrogenistein and 6′-OH-*O*-desmethylangolensin (Joannou *et al.* 1995) (Figure 2.13). Recently, Heinonen *et al.* (2002) reported that glycitein also underwent reductive metabolism to form structures 5′-OMe-*O*-desmethylangolensin and the equol analogue of glycitein (6′-OMe-equol) (Figure 2.14). Consequently, previous studies have discovered that there is a large inter-individual variability in plasma isoflavone concentrations and urinary recovery of isoflavones (Xu *et al.* 1994; King & Bursill, 1998; Lu & Anderson, 1998; Richelle *et al.* 2002), possibly the result of considerable differences in intestinal microbial composition between subjects which would ultimately influence metabolic activity and the extent of isoflavone biotransformation.

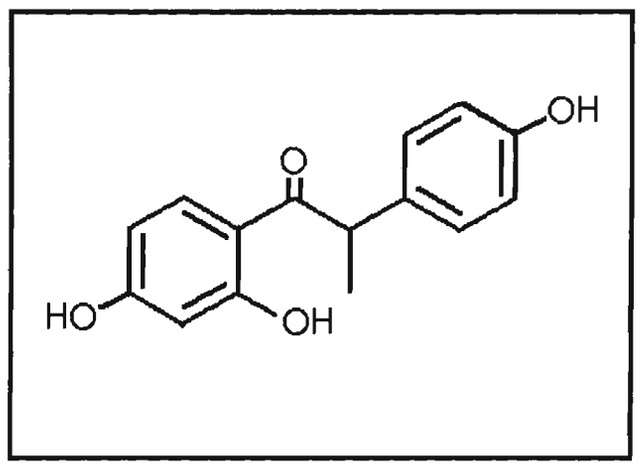

Figure 2.12 Chemical structure of daidzein metabolite *O*-desmethylangolensin. Adapted from Joannou *et al.* (1995)

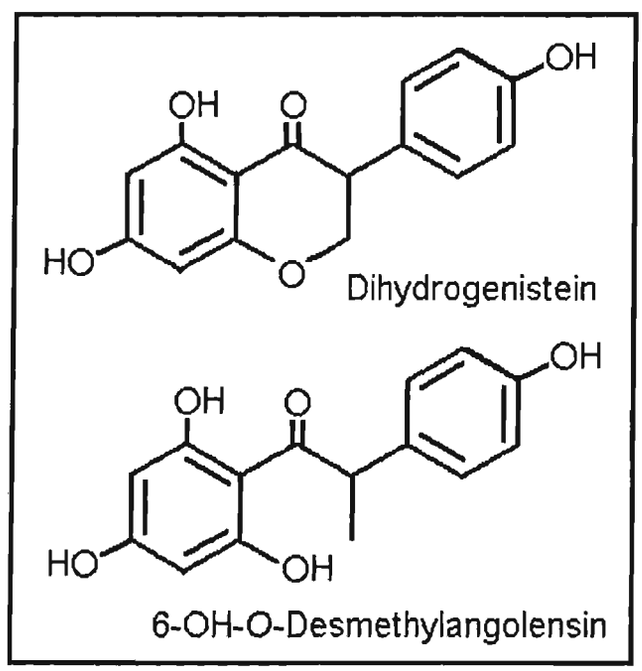

Figure 2.13 Chemical structures of metabolites of genistein. Adapted from Joannou *et al.* (1995).

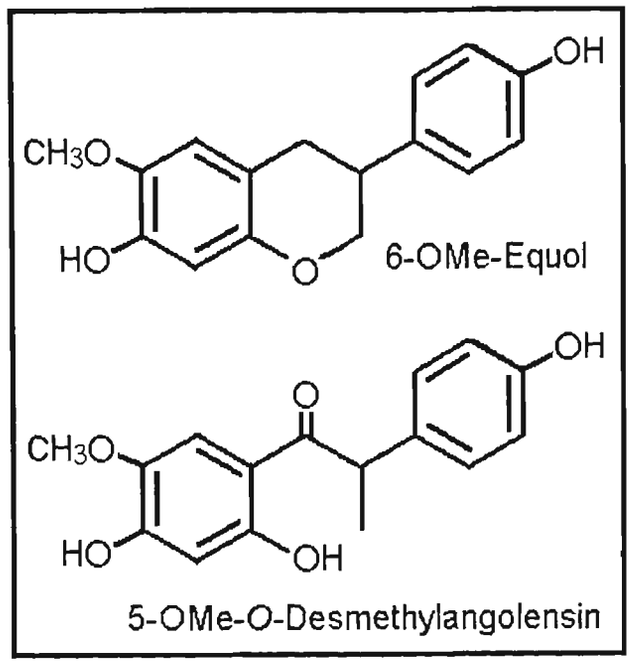

Figure 2.14 Chemical structures of metabolites of glycitein. Adapted from Heinonen *et al.* (2002).

After absorption, isoflavones are candidates for rapid biotransformation at hydroxyl groups by mammalian UDP-glucuronosyltransferases and sulfotransferases in the intestinal mucosa, liver and other organs, and this is due to their phenolic nature (Hendrich, 2002). As a result, glucuronide, sulfate and sulfoglucuronide conjugates of isoflavones are found in human urine (Adlercreutz *et al.* 1993, 1995). Even though the biological activities of conjugated isoflavones have not been well studied, an *in vitro* study by Zhang *et al.* (1999) found that daidzein and genistein glucuronides exhibited weak oestrogenic and human natural killer cell activation activities within the range of 0.1 to 10 μmol per litre. In a pharmacokinetic study, Zhang *et al.* (2003) found that the percentages of daidzein and genistein glucuronides were 73% and 71% of total daidzein and genistein excreted in urine, and 62% and 53% of total daidzein and genistein present in plasma, respectively. Only about one-fifth of circulating isoflavones were in an aglycone configuration (Zhang *et al.* 2003). Hence, glucuronide conjugates of isoflavone appear to predominate *in vivo* and possibly contribute to the observed physiological effects of isoflavones.

2.5.9 Equol

Equol, the exclusive product of intestinal bacterial metabolism of daidzein and its conjugates, is a non-steroidal compound possessing oestrogenic activity. The chemical name for equol is 7-hydroxy-3-(4′-hydroxyphenyl)-chroman and its structure is shown in Figure 2.15. The main dietary origin of equol in humans is soy foods, because they are an abundant source of daidzein and its glucosidic conjugates (Setchell *et al.* 2002*b*). In recent times, the presence of equol in human plasma and urine has aroused considerable interest amongst the scientific community researching the potential health benefits of soy isoflavones because of the wide range of biological properties of equol. An *in vitro* study by Morito *et al.* (2001) found that the binding affinity of equol for human ERα and ERβ was greater than that of daidzein and similar to the binding affinity of genistein. However, equol induced transcription more strongly than any other isoflavone, especially with ERα (Morito *et al.* 2001). *In vivo*, Nagel *et al.* (1998) showed that 49.7% of equol circulates in an unconjugated form, considerably greater than the proportion of unconjugated daidzein (18.7%), which may enhance the oestrogenic activity of equol in humans because it is the unconjugated fraction that is available for ER occupancy. In addition to its oestrogenic properties, *in vitro* studies have also found that equol exhibits greater antioxidant activity than all other isoflavones (Arora *et al.* 1998; Mitchell *et al.* 1998).

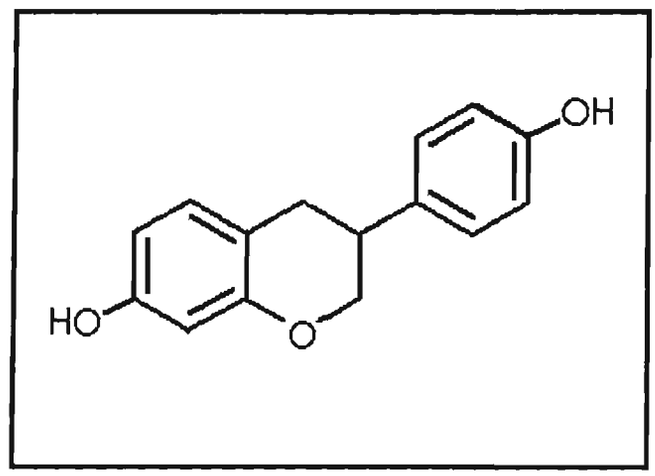

Figure 2.15 Chemical structure of daidzein metabolite equol. Adapted from Heinonen *et al.* 2002).

According to plasma and urinary pharmacokinetic studies, only about 30 to 40% of the adult population is able to synthesise equol after ingesting soy foods or isoflavone supplements (Kelly *et*

al. 1993; Hutchins *et al.* 1995; Lampe *et al.* 1998; Lu & Anderson, 1998; Rowland *et al.* 2000; Setchell *et al.* 2003). The reason for these two distinct subpopulations is unclear at this point in time, but may be due to intestinal microbial composition and its influence on intestinal enzyme systems involved in metabolic activity. Those subjects able to synthesise equol are categorised as 'equol-producers', which is generally defined as someone who excretes >1000 nmol of equol per litre of urine or has a plasma equol concentration of >83 nmol per litre after the consumption of soy foods (Setchell *et al.* 2002*b*). In a review of dietary intervention studies, Setchell *et al.* (2002*b*) stated that maximal clinical responses to soy protein diets were observed in people who were 'equol-producers', proposing that the clinical effectiveness of soy protein in cardiovascular, bone and menopausal health may be a function of the ability to biotransform daidzein and its conjugates into the more oestrogenically potent metabolite equol. Ingestion of viable bifidobacteria is known to effectively modulate intestinal microbial balance and alter intestinal metabolic activity, especially in the ileum and colon (Gomes & Malcata, 1999). However, previous studies have not investigated whether the consumption of fermented soymilk containing viable bifidobacteria stimulates the intestinal formation of equol in a group of postmenopausal women (see Chapter 8.0).

2.5.10 Isoflavones and human health

Due to their oestrogen-like activity, isoflavone phytoestrogens may play an important role in the prevention and possibly the treatment of hormone-dependent disorders, including cancer, cardiovascular disease, osteoporosis and menopausal symptoms (Adlercreutz, 1998; Setchell, 1998; Wagner *et al.* 2001; Adlercreutz, 2002; Kronenberg & Fugh-Berman, 2002). However, there have also been concerns raised about the potential adverse effects of isoflavones, especially when ingested by men (Sharpe & Skakkebaek, 1993; Santti *et al.* 1998), infants (Fort *et al.* 1990; Murphy *et al.* 1997; Fitzpatrick, 1998*a*; Zung *et al.* 2001), breast cancer patients (Petrakis *et al.* 1996; Hargreaves *et al.* 1999), and men and women with thyroid conditions (Madej *et al.* 2002). Isoflavones are considered as possible endocrine disruptors because of their oestrogenicity and therefore could negatively affect the natural growth and development of infants, harm the reproductive health of males, and promote breast cancer in women.

The consensus amongst researchers is that caution should be exercised when taking isoflavones because of their potential risks. In a recent review by Munro *et al.* (2003), it was concluded that the available scientific evidence supports the safety of isoflavones as typically consumed in diets based on soy or containing soy products, when epidemiological, clinical and *in vitro* studies are viewed in their entirety. In contrast, an earlier review by Fitzpatrick (1998*b*), which cited some of the same references as were used subsequently by Munro *et al.* (2003), concluded that it would make more sense to prohibit the addition of soy isoflavones to foods given the current state of knowledge. Nevertheless, in recent safety and pharmacokinetic studies on healthy men and postmenopausal women, it was found that a single dose of isoflavone aglycone, even at levels that exceeded normal dietary intake, had minimal clinical toxicity, with pharmacokinetic data suggesting that chronic dosing at 12 to 24 h intervals would not lead to progressive accumulation of isoflavones (Bloedon *et al.* 2002; Busby *et al.* 2002). What complicates matters about the safety of isoflavone ingestion is that up until now a clear dose-effect relationship has not been determined from animal and human studies; hence, a recommended daily intake is unknown. Most clinical studies adopt isoflavone dosage levels in the range typically ingested by Asian populations, which can vary between 20 and 100 mg per day (Brouns, 2002). Primarily, this is to determine whether these dosages of a typical soy-containing diet influence health status. In addition, isoflavone dosage levels of Asian peoples are widely implemented in research because they supposedly minimise concerns about any unexpected detrimental effects resulting from isoflavone intake, considering the lack of evidence to indicate that isoflavone-rich diets adversely affect the health of Asian populations.

Considering the body of knowledge to date, some age/gender groups may be less susceptible to the risks and more suited to the intake of phytoestrogens (e.g. healthy women in their menopausal years) than others. Consequently, it appears that a considerable amount of the research effort which is currently centred on phytoestrogens, including this project, seeks to determine if phytoestrogens may be a viable alternative to conventional oestrogen therapies for postmenopausal women by delivering a bone sparing and atheroprotective effect.

2.5.11 Menopause, hormonal status and the potential for isoflavone treatment

Menopause is the final cessation of menstruation and is due to a progressive decline in ovarian function. This results in a reduction in the production of oestrogen which initiates subtle rearrangements in the hormonal activity of the glands that control the reproductive function. The decrease in the output of oestrogen disturbs the neurovascular mechanism of the hypothalamus and probably initiates the vasomotor changes that provoke the 'hot flushes' associated with menopause. Furthermore, the metabolism of the pituitary gland is altered affecting the secretion of follicle stimulating hormone (FSH) and luteinising hormone (LH). In women, FSH stimulates the growth of ovarian follicles and, in the presence of LH, promotes secretion of oestrogens by the maturing follicles. During menopause, circulating levels of both FSH and LH become elevated as the failure of ovarian oestrogen production eliminates the negative feedback effect on the pituitary gland (Whitley *et al.* 1996).

Sex hormone binding globulin (SHBG) is a glycoprotein produced by hepatocytes which attaches itself to circulating oestrogen and prevents it from binding to ER sites. Only a small proportion of oestrogens (< 2%) are transported in their free form and it is only the unbound oestrogens that are thought to be biologically active and taken up by tissues (Tham *et al.* 1998). Changes in total oestrogen concentration result in relatively small changes in the size of the free oestrogen fraction, whereas changes in SHBG concentration result in relatively large changes in the amount of free and bound oestrogen, affecting hormonal status and the maintenance of hormone-dependent tissues (Tham *et al.* 1998). *In vitro*, isoflavones have shown to increase the synthesis and secretion of SHBG by human HepG2 hepatoblastoma cells (Mousavi & Adlercreutz, 1993; Loukovaara *et al.* 1995). Furthermore, phytoestrogens bind poorly to SHBG, circumventing the mechanism that limits steroid cell uptake (Martin *et al.* 1996; Nagel *et al.* 1998).

Conjugated oestrogens initiate rapid biological changes in postmenopausal women, including reductions in LH and FSH and increases in SHBG (Geola *et al.* 1980; Helgason, 1982). Oestrogen replacement therapy (ERT) is a widely used remedy for relief of menopausal symptoms. Over the past decade, the rapidly growing body of knowledge on the potential health benefits of

phytoestrogens has influenced many women in their menopausal years (especially of Western populations) to turn to soy isoflavones as a natural alternative to ERT and hormone replacement therapy (HRT) because of their undesirable side effects, such as a proposed increase in the risk of breast and endometrial cancer (Brzezinski & Debi, 1999; Wade *et al.* 1999; Wagner *et al.* 2001; Rossouw *et al.* 2002). Epidemiological studies have provided some evidence of a potential association between isoflavones and plasma hormones in postmenopausal women, with urinary isoflavones correlating positively with SHBG (Adlercreutz *et al.* 1987).

Clinical studies on postmenopausal women from Western populations have also been carried out to assess the hormonal effects of isoflavone intake. In a study of 97 postmenopausal women, Baird *et al.* (1995) reported that serum FSH and LH did not decrease significantly nor did SHBG increase in women on a soy food supplemented diet for 4 weeks, accompanied by very little change in endogenous estradiol concentrations. Baird *et al.* (1995) concluded that approximately 165 mg isoflavone per day (derived from soy foods) for 4 weeks showed no oestrogenic effects on the liver and pituitary of postmenopausal women. In a randomised crossover study of 18 postmenopausal women that followed, Duncan *et al.* (1999) investigated the hormonal effects of three soy powders providing different dosages of isoflavones, at approximately 7.1 (control), 65 (low-dose) and 132 (high-dose) mg per day. After 37, 65 and 93 days of treatment, Duncan *et al.* (1999) found that the high- and low-isoflavone diets provided modest hormonal effects compared to the control diet. This included small but significant changes in SHBG and FSH ($P<0.05$), but they concluded that these adjustments were probably not of physiological importance (Duncan *et al.* 1999). Pino *et al.* (2000) carried out a 10-week feeding study on 20 postmenopausal women investigating the oestrogenic effects of soymilk (30 g per day) and found that the mean concentration of plasma SHBG significantly increased after treatment ($P<0.05$), correlating with a significant increase in the total concentration of plasma isoflavones ($P<0.001$). Those women who had circulating isoflavone levels greater than 0.6 μmol per litre showed significant increases in their SHBG levels of at least 30% (Pino *et al.* 2000). However, Pino *et al.* (2000) reported no significant changes in estradiol or FSH after soymilk treatment ($P>0.05$). In two of the most recent studies on postmenopausal women, Persky *et al.* (2002) and Nicholls *et al.* (2002) investigated the hormonal effects of ingesting 56 to

90 mg isoflavone per day. Persky *et al.* (2002) conducted a longer term study of 6 months and reported that soy protein did not alter steroid hormone values, with no significant differences in the concentrations of endogenous oestrogens and FSH ($P>0.05$). Nicholls *et al.* (2002) only observed the secretion of LH over 10 to 14 days in both pre- and post-menopausal women and reported that soy isoflavone consumption did not affect mean baseline or peak LH concentrations, indicating a lack of oestrogen-like effect at the level of the pituitary. However, in postmenopausal subjects, mean LH secretion decreased after discontinuing soy intake, suggesting a residual oestrogenic effect (Nicholls *et al.* 2002).

On the whole, clinical trials investigating the effects of isoflavones (ingested via soy foods) on the hormonal status of postmenopausal women have showed highly variable results, with no strong evidence to suggest isoflavones decrease levels of FSH and LH, but some evidence to indicate isoflavone intake increases concentrations of SHBG. An aspect which has not been investigated up until now is the effect of administering an isoflavone aglycone-rich, probiotic soymilk on the hormonal status of postmenopausal women (see Chapter 9.0), bearing in mind that intestinal bacteria are suggested to play an important role in the bioavailability and bioactivity of isoflavones (Hendrich, 2002). Clinical studies by Baird *et al.* (1995), Duncan *et al.* (1999), Pino *et al.* (2000), Persky *et al.* (2002) and Nicholls *et al.* (2002) administered non-fermented, isoflavone glucoside-rich soy foods and this may account for the variability in results obtained from these studies, considering the large variation in intestinal microflora between individuals.

2.5.12 Effects of isoflavones on cardiovascular disease risk factors in postmenopausal women

The leading cause of death in women in industrialised nations is cardiovascular disease. The risk of cardiovascular disease greatly increases during menopause and is suggested to be due to oestrogen deficiency (Ososki & Kennelly, 2003). Lipid profiles are one of the most important factors that affect the onset of cardiovascular disease, with high serum cholesterol and triglyceride levels a contributing risk factor. Additionally, low-density lipoproteins (LDL) are composed primarily of cholesterol and therefore elevated levels of LDL also present risk for cardiovascular disease. In contrast, high-density lipoproteins (HDL) contain predominantly protein and serve as carriers of

cholesterol from cells back to the liver for recycling or disposal. Thus, blood lipid profiles comprising low levels of LDL (< 130 mg/dL), high levels of HDL (> 35 mg/dL), and a total cholesterol level of < 200 mg/dL are generally considered to reduce the risk of cardiovascular disease.

Just under a decade ago, Anderson *et al.* (1995) conducted a meta-analysis of 38 controlled clinical trials and found that when these studies were viewed in their entirety there was overwhelming evidence to suggest that soy protein intake improves serum lipid profiles. Anderson *et al.* (1995) concluded that an average of 47 g soy protein in place of animal protein significantly decreased serum concentrations of total cholesterol (9.3%), LDL-cholesterol (12.9%) and triglycerides (10.5%) in individuals with moderate to severe hypercholesterolemia ($P<0.001$), but with only a 2.4% increase in serum HDL-cholesterol ($P>0.05$). Since then, numerous clinical trials have been carried out on postmenopausal women to examine the potential cholesterol-lowering effects of ingesting isoflavone-rich soy foods. In a study of 42 postmenopausal women by Scheiber *et al.* (2001), subjects ingested 60 mg isoflavones per day for 12 weeks via three daily servings of soy foods and this resulted in a significant increase in mean levels of HDL-cholesterol (3.7%) ($P<0.05$) and caused a decrease in total cholesterol. In another study involving 12 weeks of treatment, Washburn *et al.* (1999) found that daily consumption of soy protein providing a lower dosage of isoflavone at 34 mg per day did not increase mean HDL-cholesterol but significantly decreased mean total cholesterol (6%) and mean LDL-cholesterol (7%) in 50 symptomatic perimenopausal women.

Other clinical studies have compared the possible effects of different isoflavone dosage levels on lipid profiles in postmenopausal women consuming soy foods. In a study involving 6 months of treatment, Baum *et al.* (1998) found that HDL-cholesterol concentrations significantly increased and LDL-cholesterol concentrations significantly decreased in two groups of postmenopausal women ingesting either 56 or 90 mg isoflavone per day via soy protein (when compared to the control group) ($P<0.05$), but total cholesterol was unchanged. In a crossover trial involving 93 days of daily soy protein consumption at three isoflavone dosage levels (7, 65 and 132 mg per day),

Wangen *et al.* (2001) discovered that plasma LDL-cholesterol concentrations were 6.5% lower after the consumption of the highest dosage ($P<0.02$), compared with the ingestion of the lowest dosage. Furthermore, the ratio of LDL to HDL cholesterol was 8.5% and 7.7% lower during low- and high-isoflavone diets, respectively ($P<0.02$). However, plasma concentrations of total and HDL-cholesterol were not significantly affected (Wangen *et al.* 2001). In only a 1-month feeding study, Jenkins *et al.* (2002) reported that isoflavone dosages of 10 mg and 73 mg per day via soy protein had similar effects on lipid profiles of men and postmenopausal women. Compared to the control diet of dairy foods, both soy protein diets resulted in significantly lower total cholesterol and ratios of total to HDL cholesterol and LDL to HDL cholesterol ($P<0.05$) (Jenkins *et al.* 2002). Gardner *et al.* (2001) carried out a 12-week parallel study on postmenopausal women consuming either soy protein with trace amounts of isoflavone, soy protein providing 80 mg isoflavone per day or a placebo of dairy milk protein. They discovered that there was a significantly greater reduction of LDL-cholesterol and total cholesterol in the isoflavone-rich soy protein group compared to the low-isoflavone group ($P<0.05$), but neither of these reductions was significantly different to the dairy protein group ($P>0.05$). In addition, changes in HDL-cholesterol and triglycerides were also similar between the soy and dairy protein groups (Gardner *et al.* 2001).

Apart from clinical trials investigating the cholesterol-lowering effects of isoflavone-rich soy foods, there have also been some studies on the specific effects of pure isoflavone (tablet or capsule of isoflavone extract) on lipid profiles. In studies by Nestel *et al.* (1997, 1999), Hodgson *et al.* (1998), and Simons *et al.* (2000), it was found that administering between 40 and 80 mg isoflavone per day (depending on the study) did not significantly change serum lipid profiles after treatment of 8 to 12 weeks. However, in a study involving only 4 weeks of treatment, Uesugi *et al.* (2002) reported that total serum cholesterol and LDL-cholesterol concentrations significantly reduced in Japanese postmenopausal women ingesting capsules providing 61.8 mg isoflavone per day when compared to the placebo group ($P<0.05$). In a longer term study of 6 months, Clifton-Bligh *et al.* (2001) found that the administration of red clover isoflavones (tablet form) at dosages of 28.5, 57 and 85.5 mg per day resulted in a significant rise in HDL-cholesterol levels (15.7 to 28.6%) in 46 postmenopausal women living in Australia. In contrast, Dewell *et al.* (2002) reported that a dosage

of 150 mg isoflavone per day in a tablet form did not significantly alter serum lipoproteins in 20 postmenopausal women (living in the US) after 6 months of supplementation, in comparison to the placebo group (n=16). Considering the results from the study by Uesugi *et al.* (2002), postmenopausal women from Asian populations may be more sensitive to the biological effects of isoflavone on lipid profiles than women from Western communities, possibly due to the composition of their intestinal microflora influencing the metabolism and bioavailability of isoflavones.

Taking into account the results from previous studies, isoflavones may be more effective at improving lipid profiles of postmenopausal women when ingested via soy foods than a tablet form, possibly because they work in conjunction with other cholesterol lowering compounds within the soy food matrix such as saponins, phytates and amino acids. Alternatively, viable *Bifidobacterium* sp. commonly found in probiotic yoghurts and fermented dairy milks have also shown to reduce total serum cholesterol in hypercholesterolemic human subjects ingesting approximately 10^9 viable cells per gram on a daily basis (Ballongue, 1993) as well as showing potential hypocholesterolemic actions *in vitro* (Gomes & Malcata, 1999). Nevertheless, there have been no studies investigating the effects of the concomitant ingestion of viable bifidobacteria and isoflavones (via a fermented soymilk) on the lipid profiles of postmenopausal women (see Chapter 9.0).

Lipoprotein(a) [Lp(a)] is a cholesterol-carrying particle in the blood that is structurally similar to LDL, with the addition of the apoprotein(a) moiety. Scientific evidence has indicated that Lp(a) is an independent risk factor for coronary heart disease (Scanu, 1992). Lp(a) levels in the blood are not responsive to many of the conventional approaches used to lower LDL-cholesterol levels, including changes in diet and lifestyle and/or the ingestion of pharmaceutical products. However, studies by Kim *et al.* (1994) and Shewmon *et al.* (1994) found that HRT lowered Lp(a) levels in postmenopausal women. Clinical studies on isoflavones and postmenopausal women have found very little evidence to indicate that soy protein intake lowers Lp(a) levels (Teede *et al.* 2001; Wangen *et al.* 2001; Tonstad *et al.* 2002). In a study by Teede *et al.* (2001), daily soy protein intake actually had a potential adverse affect, by significantly increasing Lp(a) levels after 3 months of

treatment ($P<0.05$). Hodgson *et al.* (1998) administered a daily dose of 55 mg isoflavone via a tablet for a period of 8 weeks and found that this treatment did not significantly change Lp(a) concentrations in postmenopausal women when compared to a placebo group. However, there have been no previous attempts to investigate the effects of modulating intestinal microbial balance via the ingestion of viable bifidobacteria, potentially enhancing the bioavailability of isoflavones derived from soymilk, on Lp(a) concentrations in postmenopausal women (see Chapter 9.0).

2.5.13 Isoflavones, bone health and the prevention of osteoporosis in postmenopausal women

Oestrogen deficiency in postmenopausal women is associated with elevated bone resorption caused by a rise in osteoclast numbers. Oestrogen either directly or indirectly suppresses or regulates the production of cytokines which regulate osteoclast generation (Watanabe *et al.* 2002). Elevated bone resorption in postmenopausal women increases the fragility of bone and its susceptibility to fractures, known as the degenerative bone disease osteoporosis. Due to their oestrogenic activity, isoflavones are currently under the spotlight as a possible alternative treatment for osteoporosis for women in their menopausal years considering the numerous reports of adverse cancer-related effects associated with traditional HRT. The potential for isoflavones to prevent bone loss and osteoporosis is supported by *in vitro* evidence indicating that genistein and daidzein have a greater affinity for ERβ than ERα, with the process of bone formation stimulated by oestrogen through ERβ (Kuiper *et al.* 1998).

The incidence of osteoporosis-related fractures is considerably lower amongst postmenopausal women from Asian countries than in most Western communities, possibly due to the isoflavone-rich soybeans and vegetables consumed in large quantities in the Asian diet (Tham *et al.* 1998). In a study on Southern Chinese postmenopausal women (n=357), Mei *et al.* (2001) reported that those women with a habitually high intake of dietary isoflavone were associated with higher bone mineral density values at both the spine and hip region. Additionally, women with the highest intake of isoflavone had significantly lower levels of bone resorption marker urinary N-telopeptide ($P<0.05$). According to Mei *et al.* (2001), high isoflavone intake may help to reverse the state of secondary hyperparathyroidism associated with oestrogen withdrawal and hence lower the rate of bone

turnover in postmenopausal women. In a recent double-blind, placebo-controlled trial carried out by Chen *et al.* (2003), 203 Chinese postmenopausal women were randomly assigned to three treatment groups to ingest either a placebo, mid-dose of isoflavone (~40 mg per day via soy germ extracts) or a high-dose of isoflavone (~80 mg per day via soy germ extracts) for 1 year, with bone mineral density and content of the whole body, spine and hip measured using dual energy x-ray absorptiometry before and after treatment. Of all the results, Chen *et al.* (2003) highlighted that women in the high-dose group had significantly higher favourable change rate in bone mineral content at the total hip and trochanter compared with the placebo and mid-dose group ($P<0.05$), but the positive effects of soy isoflavone supplementation were only observed amongst women with lower initial baseline bone mineral content (median or less). In a study of Japanese postmenopausal women, Uesugi *et al.* (2002) found that only 4 weeks of isoflavone ingestion (61.8 mg per day) significantly reduced concentrations of bone resorption markers pyridinoline and deoxypyridinoline excreted in urine ($P<0.05$), but had no affect on bone stiffness or the bone formation marker osteocalcin ($P>0.05$).

Numerous clinical studies have been carried out on postmenopausal women from Western populations to investigate the effects of isoflavones on bone condition (bone mineral density and bone mineral concentration) and bone health (markers of bone formation and resorption). In a 6-month study, Potter *et al.* (1998) found that the daily ingestion of 40 g soy protein (containing 2.25 mg isoflavone per gram) significantly increased bone mineral content and density in the lumbar spine region but not elsewhere, when compared with the control group ($P<0.05$). In a study by Alekel *et al.* (2000) of similar duration (24 weeks), percentage change in lumbar spine bone mineral content and density did not decline from baseline in perimenopausal women ingesting an isoflavone dosage of 80.4 mg per day (via soy protein), with significant losses in bone mineral content and density occurring in the control group ($P<0.05$). However, Alekel *et al.* (2000) could not correlate the positive effects on bone mineral content and density to changes in bone turnover markers serum bone-specific alkaline phosphatase (bone formation) and urinary N-telopeptide (bone resorption), as these markers were not affected by soy isoflavone supplementation. Similarly, Wangen *et al.* (2000) reported that ingesting up to 130 mg isoflavone per day (via soy protein) for 3 months had very

little effect on bone markers of formation and resorption in 17 postmenopausal women. Nevertheless, Scheiber *et al.* (1999) used a larger study group of 50 postmenopausal women, each consuming a dosage of 60 to 70 mg isoflavone per day via soymilk for 12 weeks, and found that this significantly reduced bone resorption marker urinary N-telopeptide and increased bone formation marker serum osteocalcin ($P<0.05$).

Given the slow rate of bone turnover and the variability in data from previous bone-related studies on the effects of isoflavone supplementation for 3 to 6 months, Lydeking-Olsen *et al.* (2002) conducted a 2-year bone study on postmenopausal women. They reported that after 2 years of daily soymilk consumption administering 50 mg isoflavone per day, there was a 1.1% and 2% increase in lumbar spine bone mineral density and content, respectively (Lydeking-Olsen *et al.* 2002). It was concluded that isoflavone consumed consistently for a prolonged period protected against bone loss in postmenopausal women, considering that the group ingesting soymilk with negligible amounts of isoflavones showed significant decreases in lumbar spine bone mineral density and content of 4.0% and 4.3%, respectively, over the 2-year period ($P<0.01$) (Lydeking-Olsen *et al.* 2002). Studies by Scheiber *et al.* (1999) and Lydeking-Olsen *et al.* (2002) reported positive effects on bone turnover and condition, respectively, as a result of soymilk consumption. The effects of isoflavone-rich soymilk on bone turnover in postmenopausal women may be enhanced if it is fermented by bifidobacteria (see Chapter 9.0), by possibly providing a greater proportion of isoflavone in a bioactive aglycone configuration and viable populations of probiotic bacteria able to modulate intestinal microflora.

2.6 Probiotic bacteria

The word 'probiotic', originating from the Greek word 'for life', is used to classify viable microorganisms that exhibit a beneficial effect on the health of the host upon ingestion by improving the balance of microflora in the gut (Fuller, 1989; Gomes & Malcata, 1999). The main genera of microorganisms which have been associated with probiotic properties according to *in vitro* and human clinical studies are *Lactobacillus*, *Bifidobacterium* and yeast *Saccharomyces*. However, health benefits imparted by probiotic bacteria are strain-specific and not species- or

genus-specific (Playne, 2002). Probiotic bacteria are typically ingested via fermented dairy products (e.g. yoghurt and fermented milks), soy foods (e.g. soy yoghurt) or freeze-dried probiotic capsules. To obtain the desired therapeutic effects of probiotic bacteria, it has been suggested that these microorganisms be present in food at high levels of $\geq 10^6$ viable cells per gram, to compensate for the possible reduction in numbers during passage through the stomach and intestine (Gomes & Malcata, 1999; Shah, 2000).

When probiotic bacteria are ingested via foods, other constituents may also enhance the effects of these microorganisms on intestinal microflora, such as oligosaccharides, inulin and resistant starch due to their known prebiotic effects (Playne & Crittenden, 1996; Playne, 2002). Furthermore, combining two or three probiotic strains or a combination of probiotic and starter culture strains is common in the manufacture of probiotic foods, influencing the viability and health effects of probiotic bacteria (Shah, 2000; Playne, 2002). In most cases, different bacterial strains (probiotic and starter culture) from various genera act in synergy during fermentation, consequently improving the sensory, nutritional and functional (probiotic) properties of the final product. Some examples of this practice commonly used in the manufacture of yoghurt include the combination of probiotic cultures *Lactobacillus acidophilus* and *Bifidobacterium* sp. (referred to as AB cultures) or *L. acidophilus*, *Bifidobacterium* sp. and yoghurt bacterium *Streptococcus thermophilus* (referred to as ABT cultures) (Shah, 2000). In probiotic yoghurt production, the inclusion of *S. thermophilus* improves the sensory and physical properties of the final product and is also able reduce the pH of the product to 4.5 or lower (to meet legal requirements) over a short fermentation period (4 to 6 h). Even though probiotic strains like *L. acidophilus* and *Bifidobacterium* sp. produce organic acids during growth, they are generally slow growing and need long fermentation times of up to 20 h to reduce the pH level to below 4.5 when used as sole starters (Saxelin *et al.* 1999).

Of the commercially available strains of probiotic bacteria, there are four strains which have the most published clinical data on their potential health benefits, including *Lactobacillus rhamnosus* GG (Valio), *Saccharomyces cereviseae* Boulardii (Biocodex), *Lactobacillus paracasei* Shirota (Yakult) and *Bifidobacterium animalis* BB12 (formerly known as *B. lactis* BB12; Chr Hansen)

(Playne, 2002). On the whole, the potential health benefits associated with the ingestion of these probiotic bacteria includes the treatment and prevention of rotaviral-, traveller's-, antibiotic-associated- and *Clostridium difficile*-diarrhoea, the lessening of lactose intolerance, reduction of constipation, modulation of immune response, alleviation of atopic dermatitis symptoms in children, and elimination of *Helicobacter pylori* (organism responsible for gastric ulcers) (Isolauri, 2001; Isolauri *et al.* 2001; Playne, 2002). In a review of clinical data by Playne (2002), it was stated that there is also emerging evidence to indicate that probiotic bacteria may play a role in the prevention of bowel and bladder cancer, irritable bowel syndrome and inflammatory bowel diseases (e.g. Crohn's disease), and arthritis. Furthermore, St-Onge *et al.* (2000) stated that evidence from animal and human studies suggests a moderate cholesterol-lowering effect associated with the consumption of fermented dairy products containing probiotic strains, possibly reducing the risk of cardiovascular disease. In contrast, a review by Salminen *et al.* (1998) on the safety of probiotics suggested that consumers of probiotic bacteria, in theory, may be at risk of systemic infections, deleterious metabolic activities causing disease, immunomodulation and gene transfer. To date, no cases of clinical infections or diseases have been traced to the ingestion of probiotic bacteria. However, the review by Salminen *et al.* (1998) found that three cases of fungemia were reported as a result of oral treatment with yeast *Saccharomyces boulardii*, which were all resolved with anti-fungal therapy.

2.6.1 Genus *Bifidobacterium*

Since their first isolation from faeces of breastfed infants in 1899 by Tissier of the Pasteur Institute, the genus *Bifidobacterium* has gradually evolved to include up to 31 species to date (Figure 2.16). Eleven of these species have been isolated from humans (adults and/or infants), and the remainder from intestinal tracts or rumen of animals (Tannock, 1999; Gomes & Malcata, 1999). Other known sources of *Bifidobacterium* species include honey-bees (Shah & Lankaputhra, 2002), wastewater and fermented milk (Gomes & Malcata, 1999). Presently, five species of *Bifidobacterium* have attracted attention in the dairy industry for manufacturing probiotic milk products: *B. adolescentis*, *B. bifidum* (most commonly used), *B. breve*, *B. infantis* and *B. longum* (Shah & Lankaputhra, 2002), all of which have been isolated from humans.

Species	Isolated from
B. adolescentis	Human (adults)
B. angulatum	Human
B. animalis	Animal
B. asteroides	Animal
B. bifidum	Human (infants and adults)
B. boum	Animal
B. breve	Human (infants)
B. catenulatum	Human (infants and adults)
B. choerinum	Animal
B. coryneformes	Animal
B. cuniculi	Animal
B. denticolens	Animal
B. dentium	Human (infants)
B. gallicum	Human
B. gallinarum	Animal
B. globosum	Human
B. indicum	Animal
B. infantis	Human (infants)
B. inspinatum	Animal
B. longum	Human (infants and adults)
B. magnum	Animal
B. merycicum	Animal
B. minimum	Animal
B. pseudocatenulatum	Human (infants and adults)
B. pseudolongum	Animal (cattle and pigs)
B. pullorum	Animal
B. ruminantium	Animal
B. saeculare	Animal
B. subtile	Animal
B. suis	Animal
B. thermophilum	Animal (cattle and pigs)

Figure 2.16 List of species of the genus *Bifidobacterium* and their sources. Adapted from Tannock (1999), Gomes & Malcata (1999) and Shah & Lankaputhra (2002).

Bifidobacteria are predominant members of the human gastrointestinal microflora, especially in the ileum and colon where they are found at populations of 10^3 to 10^7 and 10^8 to 10^{12} CFU per gram of intestinal contents, respectively (Orrhage & Nord, 2000; Shah & Lankaputhra, 2002). When present in sufficient numbers in the intestinal tract, bifidobacteria create a healthy equilibrium between beneficial and potentially harmful microorganisms (Shah & Lankaputhra, 2002). Populations of bifidobacteria in the intestine decrease with increasing age. Bifidobacteria are the dominant bacterial species in the intestine of infants, alongside lower populations of coliforms, enterococci and clostridia. In adults, bifidobacteria become the second or third most abundant genus, with

intestinal microflora generally consisting of Bacteroidaceae (up to 86% of the total flora), *Eubacterium* (6 to 19%), bifidobacteria (6 to 36%), Peptococcaceae (2 to 14%), Enterobacteriaceae (trace to 5.3%), streptococci and lactobacilli (trace) (Finegold *et al.* 1983; Shah & Lankaputhra, 2002). Furthermore, the profile of *Bifidobacterium* species changes; *B. infantis* and *B. breve*, typically associated with infants, are replaced by *B. adolescentis* in adults, with *B. longum* staying as a lifelong inhabitant (Mitsuoka, 1990). This change in *Bifidobacterium* profile is most probably influenced by dietary intake and by the host's physiology (Kurmann & Rasic, 1991; Modler, 1994). In the elderly, the populations of bifidobacteria decrease further due to less secretion of gastric juices, this is accompanied by increases in the numbers of coliforms, enterobacteria and clostridia (Mitsuoka, 1984; Shah & Lankaputhra, 2002).

Bacteria of the genus *Bifidobacterium* present a globally bacillar form, show Gram-positive staining, are non-motile, non-spore forming, and catalase-negative anaerobes. They have various shapes including short, curved rods, club-shaped rods and bifurcated Y-shaped rods (Ballongue, 1993; Gomes & Malcata, 1999). Bifidobacteria are phylogenetically grouped in the actinomycete branch of Gram-positive bacteria, that is, characterised by a high guanine plus cytosine (G + C) content, which varies from 54 to 67 mol% (Sgorbati *et al.* 1995). They are saccharolytic organisms that exclusively degrade hexoses by the fructose-6-phosphate pathway (Scardovi & Trovatelli, 1965), with the fermentation of two moles of glucose generally leading to the production of three moles of acetate and two moles of lactate without generation of CO_2 (Figure 2.17). The key enzyme involved in this glycolytic pathway is fructose-6-phosphate phosphoketolase (F6PPK), which is used as a taxonomic character in identification of the genus (Gomes & Malcata, 1999). Besides glucose, all bifidobacteria from human origin are able to utilise galactose and lactose as carbon sources (Krzewinski *et al.* 1996; Gomes & Malcata, 1999). Additionally, studies have shown that *Bifidobacterium* are able to metabolise complex carbohydrates amylose and amylopectin (Crociani *et al.* 1994) and oligosaccharides raffinose and stachyose (Scalabrini *et al.* 1998; Hou *et al.* 2000; Desai *et al.* 2002), potentially utilising their monomers as growth substrate. Bifidobacteria produce numerous intracellular and extracellular saccharolytic enzymes during growth, including β-glucosidase, β-galactosidase, α-galactosidase and α-glucosidase (Tochikura *et al.* 1986; Desjardins

& Roy, 1990). The types and levels of saccharolytic enzymes produced depend on the strain of *Bifidobacterium* rather than the species.

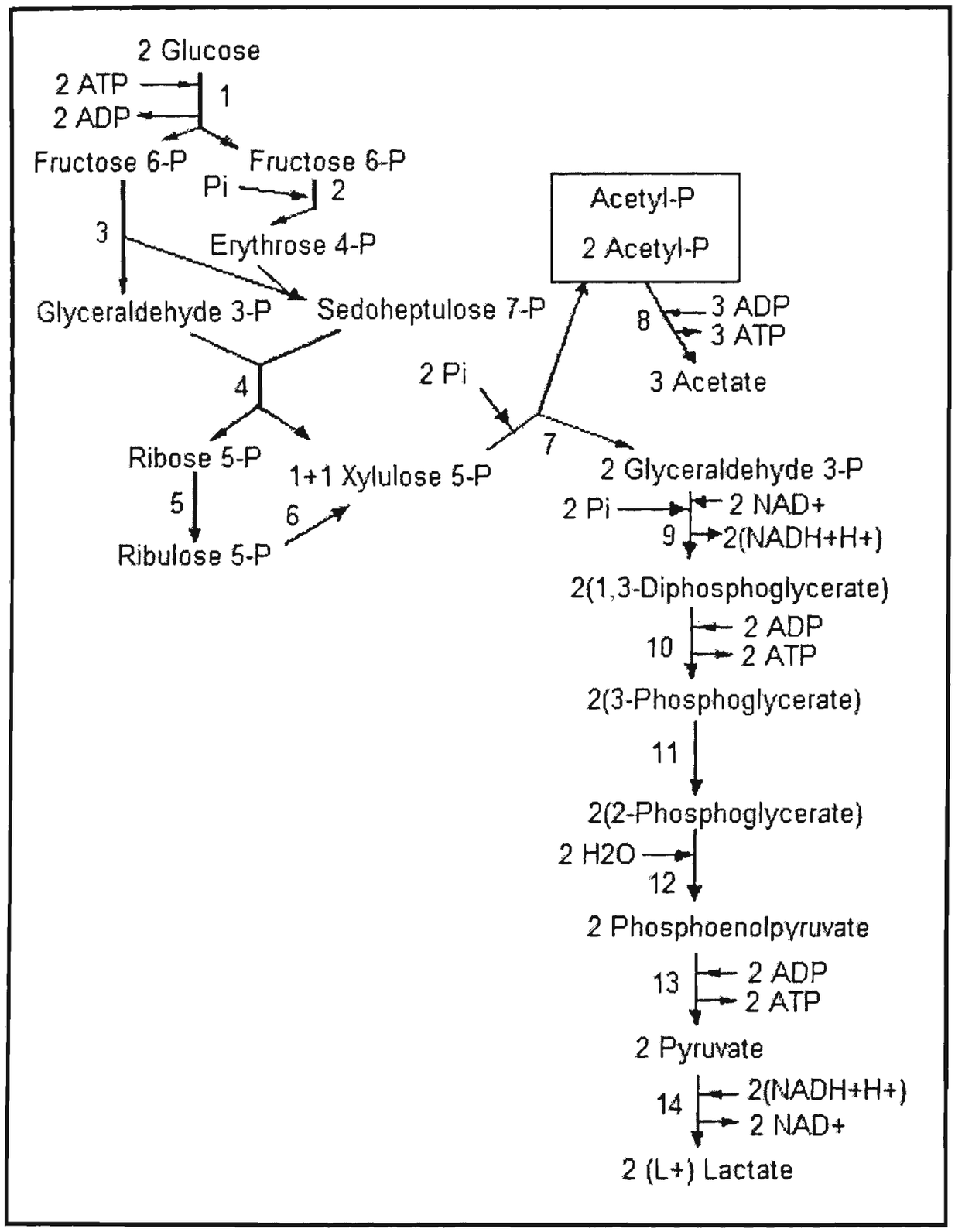

Figure 2.17 Carbohydrate metabolic pathway of *Bifidobacterium*. (1) hexokinase and glucose-6-phosphate isomerase; (2) fructose-6-phosphate phosphoketolase; (3) transaldolase; (4) transketolase; (5) ribose-5-phosphate isomerase; (6) ribulose-5-phosphate epimerase; (7) xylulose-5-phosphate phosphoketolase; (8) acetate kinase; (9) homo-fermentative pathway enzymes; (10) L(+)-lactate dehydrogenase; (11) phosphoroclastic enzyme; (12) formate dehydrogenase; (13) alcohol dehydrogenase; (14) homo-fermentative pathway enzymes. Adapted from Ballongue (1993).

The optimum pH for growth of bifidobacteria is 6.0 to 7.0, with virtually no growth at pH 4.5 to 5.0 or below or at pH 8.0 to 8.5 or above (Gomes & Malcata, 1999). Below pH 4.1, most species of bifidobacteria die within a week even at 4°C, and below pH 2.5 most species die within 3 h (Shah & Lankaputhra, 2002). With respect to temperature, bifidobacteria can grow in the range of 25 to 45°C, with the optimum growth temperature between 36 and 38°C for strains of human origin and 41 to 43°C for those of animal origin (Ballongue, 1993). Growth of bifidobacteria does not occur below 20°C; hence, refrigerated storage effectively maintains the viable populations of bifidobacteria attained during fermentation. Apart from optimum growth temperature and pH, numerous 'bifidogenic' or 'bifidus' factors (usually of a carbohydrate nature) have been identified to stimulate the growth of bifidobacteria in synthetic mediums, milk and in the intestinal tract, including *N*-acetyl-D-glucosamine and lactulose from human milk, and the oligosaccharides raffinose, stachyose and inulin (known as prebiotics). Furthermore, the addition of the sulphur-containing amino acid cysteine to milk appears to enhance the growth of bifidobacteria by possibly acting as an additional nitrogen source and lowering the redox potential of the growth environment (Dave & Shah, 1997). Bifidobacteria lack proteolytic activity when grown in milk and this slows their growth. Hence, additional nitrogen sources in the form of peptides or amino acids are shown to enhance the viability of bifidobacteria (Shah, 2000; Shah & Lankaputhra, 2002).

2.6.2 Metabolic activities of bifidobacteria in soymilk

Numerous studies have investigated the growth and metabolic activity of *Bifidobacterium* sp. during the fermentation of whole bean soymilk, in terms of protein hydrolysis, metabolism of oligosaccharides (raffinose and stachyose) and aldehydes (hexanal and pentanal), utilisation of simple sugars and their effects on growth, and the production of organic acids (acetic and lactic acid) (Kamaly, 1997; Scalabrini *et al.* 1998; Chou & Hou, 2000; Hou *et al.* 2000; Desai *et al.* 2002; Wang *et al.* 2002; Wang *et al.* 2003). According to these studies, soymilk is a suitable growth medium for bifidobacteria, and this may be predominantly due to the rich levels of oligosaccharides raffinose and stachyose, which are known to stimulate the growth of this genus (classified as 'bifidogenic factors'). However, Kamaly (1997) found that *B. bifidum* and *B. longum* showed better

growth in MRS broth (de Mann *et al.* 1960) and reconstituted skim milk (RSM) than whole bean soymilk, even though these strains showed greater proteolytic activity when grown in soymilk than RSM (according to free amino groups expressed as glycine equivalents, μM per mL). Similarly, Hou *et al.* (2000) reported that protein hydrolysis significantly increased during fermentation of soymilk by *B. infantis* (CCRC 14633) and *B. longum* (B6) ($P<0.05$), when expressed as the content of leucine amino equivalent (mmol per litre). Kamaly (1997) found that supplementation of soymilk with either lactose, galactose or glucose, or the addition of yeast extract, protease peptone, casitone, polypeptone or phytone (protein hydrolysates) enhanced the growth of *B. bifidum*, but had no effect on *B. longum*. In a later study, Chou & Hou (2000) also reported that supplementation of soymilk with either isomaltooligosaccharide, glucose, lactose or galactose increased the growth of *B. infantis* (CCRC 14633) and *B. longum* (B6) after 48 h of fermentation, but *B. infantis* reached its maximum population in a shorter cultivation time of 24 h when either yeast extract, peptone, tryptone or casitone were added to the soymilk. Furthermore, Kamaly (1997) found that the addition of sulphur-containing amino acid cysteine increased the populations of *B. longum* and *B. bifidum* by five times compared with soymilk without cysteine, in line with a study by Dave & Shah (1997) in which cysteine supplementation improved the viability of bifidobacteria in yoghurt production.

Several studies have investigated the metabolic effects of *Bifidobacterium* sp. on raffinose and stachyose concentrations in whole bean commercial soymilks (Scalabrini *et al.* 1998; Hou *et al.* 2000; Wang *et al.* 2003), to determine whether or not fermentation by bifidobacteria can eliminate these flatulence-causing constituents. Scalabrini *et al.* (1998) reported that *B. breve*, *B. infantis* and *B. longum* completely metabolised the concentration of raffinose (250 mg per 100 mL) in soymilk after 48 h of fermentation, but were unable to completely eliminate the concentration of stachyose (530 mg per 100 mL) after the same incubation period. The greatest reduction in stachyose concentration occurred in soymilk fermented by *B. longum*, decreasing to 190 mg per 100 mL after 48 h of fermentation (Scalabrini *et al.* 1998). In a later study, Hou *et al.* (2000) found that two different strains of *Bifidobacterium*, *B. infantis* (CCRC 14633) and *B. longum* (B6), only metabolised 30 to 40% of raffinose and 50 to 65% of stachyose in a whole bean soymilk after 48 h of fermentation, with this soymilk containing lower concentrations of raffinose and stachyose at

69.6 mg and 360.2 mg per 100 mL, respectively. According to Scalabrini *et al.* (1998), α-galatosidase activity varies considerably between different strains of the same species of *Bifidobacterium*. Hence, this influences their ability to hydrolyse α-galactosyl sugars when grown in soymilk and affects the extent to which raffinose and stachyose is reduced in the fermented product. In a more recent study, Wang *et al.* (2003) introduced the same strains of *B. infantis* and *B. longum* used by Hou *et al.* (2000) into soymilks containing either *L. acidophilus* or *S. thermophilus* and fermented these combined cultures for up to 32 h. They found that this significantly enhanced the metabolism of raffinose and stachyose after 16 and 24 h of incubation compared to soymilks fermented by either *L. acidophilus* or *S. thermophilus* as sole starters ($P<0.05$) (Wang *et al.* 2003).

Apart from metabolising oligosaccharides, Scalabrini *et al.* (1998) and Desai *et al.* (2002) reported that *Bifidobacterium* sp. metabolised the objectionable volatiles hexanal and pentanal in whole bean soymilk during fermentation. Scalabrini *et al.* (1998) discovered that pentanal (at 8.9 ppb in non-fermented soymilk) was no longer detectable in soymilk after 24 h of fermentation by *B. breve*, which was accompanied by a decrease in hexanal from 16 ppb to 4 ppb. Similarly, Desai *et al.* (2002) found that soymilk contained higher levels of hexanal (12.4 ppb) than pentanal (8.8 ppb). Furthermore, each strain of *Bifidobacterium* under analysis (*B. bifidum*, *B. infantis*, *B. longum* and *B. pseudolongum*) was able to reduce the concentrations of pentanal to non-detectable levels, but in this case after 48 h of incubation. Only *B. infantis* and *B. pseudolongum* appeared to completely metabolise the concentration of hexanal in the soymilk analysed by Desai *et al.* (2002), with non-detectable levels after 48 h of fermentation.

Organic acid by-products of the carbohydrate metabolic pathway of bifidobacteria during their growth in whole bean soymilk have also been studied by Scalabrini *et al.* (1998) and Hou *et al.* (2000). According to the frustose-6-phosphate pathway (Figure 2.17), bifidobacteria produce acetic and lactic acid in a molar ratio of 1.5 (De Vries & Stouthamer, 1967). However, fermentation studies on soymilk have shown that this varies considerably between strains of bifidobacteria (Scalabrini *et al.* 1998; Hou *et al.* 2000). Due to the undesirable 'vinegary' flavour of acetic acid, greater levels of lactic acid by-product are preferred from the fermentation of soymilk by

bifidobacteria. Scalabrini *et al.* (1998) reported that 4 strains of bifidobacteria out of a total of 27 exhibited a favourable ratio of lactic to acetic acid (> 1.7) at 12, 24 and 48 h of fermentation, including one strain of *B. breve* and *B. longum* and two strains of *B. infantis*. There was a considerable variation in the production of organic acids, with the lactic acid/acetic acid ratio varying from 0.34 to 3.80 (Scalabrini *et al.* 1998). Furthermore, variation in lactic acid/acetic acid ratio was also observed between incubation times (i.e. 12, 24 and 48 h) (Scalabrini *et al.* 1998), which was also observed in a later study by Hou *et al.* (2000). Hou *et al.* (2000) found that *B. infantis* (CCRC 14633) and *B. longum* (B6) both produced an average acetic acid/lactic acid molar ratio of 1.7, which was close to the theoretical value of 1.5. Apart from studies on the composition of organic acids in soymilk fermented by bifidobacteria, Chou & Hou (2000) assessed the effects of organic acid production on the pH levels of soymilk and the viability of bifidobacteria during refrigerated storage. In plain soymilk, growth of *B. infantis* (CCRC 14633) reduced the pH from 6.5 to 4.6 after 48 h of incubation, with the drop in pH predominantly occurring between 24 and 48 h. In contrast, the pH of plain soymilk fermented by *B. longum* (B6) only reduced from 6.5 to 6.1 after 48 h of incubation, but acid production was enhanced when the soymilk was supplemented with galactose or malt extract as the pH dropped to 4.0 after 48 h. Chou & Hou (2000) also reported that storing each of the plain and supplemented soymilks at 5°C maintained the viable populations of both strains for up to 10 days at approximately 7.8 $\log_{10}$ CFU per mL, stating that this was due to the stable pH levels during refrigerated storage causing very little loss in viability typically caused by post-acidification.

2.6.3 Bifidobacteria and human health

Bifidobacterium animalis Bb-12 is the most thoroughly investigated probiotic *Bifidobacterium* strain currently on the market. In human studies, *B. animalis* Bb-12 has shown to prevent traveller's diarrhoea (Black *et al.* 1989), treat viral diarrhoea (Saavedra *et al.* 1994), modulate intestinal microflora (Marteau *et al.* 1990), ameliorate constipation (Alm *et al.* 1993), modulate immune response (Link-Amster *et al.* 1994; Schiffrin *et al.* 1995; Fukushima *et al.* 1998), and alleviate atopic dermatitis symptoms in children (Kankaanpää *et al.* 1998). There have also been numerous reported *in vitro* effects supporting the potential disease-preventative actions of *Bifidobacterium* sp.,

including the removal of cholesterol by precipitation and assimilation (hypocholesterolemic actions) (Tahri *et al.* 1995), adhesion to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions (possible stabiliser of the gut mucosal barrier and immune enhancer) (Bernet *et al.* 1993), elimination of nitrite and metabolism of nitrosamines (anti-carcinogenic activity) (Grill *et al.* 1995), and inhibitory effects on the growth of pathogenic bacteria (protection against gastroenteritis) (Gibson & Wang, 1994; Fujiwara *et al.* 1997). However, up until now there has been no attempt to determine whether or not bifidobacteria are able to biotransform isoflavone glucosides into their bioactive aglycone forms in soymilk (see Chapters 3.0, 4.0 and 6.0), and if this enhances the bioavailability and bioactivity of isoflavones in humans (see Chapters 7.0, 8.0 and 9.0). Considering that both isoflavones and bifidobacteria have been associated with potential disease-preventative actions (from *in vivo* and *in vitro* studies), their concomitant ingestion may be of enhanced health benefit.

3.0 Enzymic Transformation of Isoflavone Phytoestrogens in Soymilk by β-Glucosidase-Producing Bifidobacteria

3.1 INTRODUCTION

Isoflavones are predominantly found in soybeans and non-fermented soy foods as biologically inactive malonyl-, acetyl- and β-glucoside conjugates, which comprise 80 to 95% of the total isoflavone concentration (Franke *et al.* 1999; Murphy *et al.* 1999; King & Bignell, 2000). With respect to the bioavailability of isoflavones, research has shown that isoflavone aglycone structures are absorbed faster and in higher amounts than their respective glucosidic conjugates in humans (Hutchins *et al.* 1999; Izumi *et al.* 2000). It has been proposed that intestinal microflora play a key role in the metabolism and bioavailability of isoflavones, as they hydrolyse the glucoside components via β-glucosidase, releasing the bioactive aglycone form (Setchell, 2000; Hendrich, 2002). However, the efficacy of microbial biotransformation and types of intestinal bacteria involved in isoflavone conversion to bioactive forms are not entirely known. Bifidobacteria are predominant members of intestinal microflora (Orrhage & Nord, 2000). Some strains of bifidobacteria are known to produce β-glucosidase (Tochikura *et al.* 1986) needed to deconjugate isoflavone glucosides to bioactive aglycones. However, transformation of isoflavones to biologically potent and bioavailable forms in soybean foods by bifidobacteria has not been studied.

The objectives of this study were to screen strains of *Bifidobacterium* for β-glucosidase activity, examine the growth of the selected strains in soymilk, and quantify the levels of isoflavone glucosides and aglycones before and after fermentation.

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3.2 MATERIALS AND METHODS

3.2.1 Bacteria

Pure cultures of four strains of *Bifidobacterium* from the Australian Starter Culture Research Centre Culture Collection (Werribee, Victoria, Australia), including *Bifidobacterium longum* CSCC 5550 (BB536), *Bifidobacterium longum* CSCC 1941 (BL1941), *Bifidobacterium pseudolongum* CSCC 1944 (BP20099) and *Bifidobacterium infantis* CSCC 1912 (BI1912) were stored at –80°C in 12% (w/v) sterile (121 °C/15 min) reconstituted skim milk supplemented with D-glucose (1% w/v), yeast extract (0.5% w/v) and glycerol (40% v/v). *Bifidobacterium animalis* Bb-12 VUP 13519 (BB12) was from the Victoria University Culture Collection (Werribee, Victoria, Australia) and stored under the same conditions. BB12 and BB536 were originally obtained from Chr Hansen Pty. Ltd. (Bayswater, Victoria, Australia) and Morinaga Milk Industry Co., Ltd. (Tokyo, Japan), respectively. BP20099, BL1941 and BI1912 were originally obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) (Highett, Victoria, Australia); BP20099 was isolated from cattle and BL1941 and BI1912 from human infants.

3.2.2 Bacterial growth media

Rehydrated MRS broth (pH adjusted to 6.7 using 5 M NaOH) and MRS agar (de Mann *et al.* 1960) containing additional 1% (w/v) D-glucose were prepared according to manufacturer instructions (Oxoid Ltd., West Heidelberg, Victoria, Australia) and autoclaved at 121°C for 15 min. Filter-sterilised L-cysteine-HCl solution (5% w/v) was also added to both the broth and agar (0.05% w/v final concentration) just prior to inoculation to lower the oxidation-reduction potential of the medium and to enhance the growth of anaerobic bifidobacteria. D-Glucose and L-cysteine-HCl were purchased from Sigma Chemical Company (Castle Hill, NSW, Australia).

3.2.3 Soymilk manufacture

Supro® 590 soy protein isolate (SPI590) (Protein Technologies International) comprising 1.2 ± 0.2 mg isoflavone per gram, and a macronutrient composition of 90 g of protein, 4 g of fat and ≤ 4 g of soluble carbohydrate per 100 g (according to manufacturer specifications) was used in the production of soymilk at a ratio of 40 g per 1 L ultra-pure distilled water. SPI590 was selected as the soy ingredient instead of whole soybeans because it simplified the manufacturing process and the effects of fermentation by bifidobacteria on soymilk made from SPI had not been studied previously. For reconstitution, distilled water was heated to 40°C prior to the addition of SPI590 powder, followed by heating and stirring at 50 to 60°C for 30 min to disperse solid particles. After cooling to room temperature, the pH was adjusted to 6.7 (using 5 M NaOH), within the optimum pH range for *Bifidobacterium* sp. (Ballongue, 1993; Gomes & Malcata, 1999). A fourteen-litre batch of soymilk was prepared in a 20-litre stainless steel pot and dispensed into twenty-four glass bottles in 500 mL and 50 mL quantities using a 100 mL Trubor® bottle top dispenser (U-Lab Pty. Ltd., Eltham, Victoria, Australia). The remaining 800 mL was used for analyses as non-fermented soymilk sample (control). The entire volume of soymilk was sterilised by autoclaving at 121°C for 15 min.

3.2.4 Assay for β-glucosidase activity and the effects of sugar addition on enzyme production

β-Glucosidase activity of the five strains of *Bifidobacterium* grown in MRS broth, and MRS broth containing additional 1% (w/v) D-glucose (MRS-gluc), 1% (w/v) lactose (MRS-lac) or 1% (w/v) D-raffinose (MRS-raf), was determined. All three sugar supplements were purchased from Sigma.

Strains were firstly activated in MRS, MRS-gluc, MRS-lac and MRS-raf broths by transferring successively on three occasions. Each incubation was at 37°C for 20 h. Subsequently, 10 mL of activated culture was inoculated in triplicate into 200 mL of each broth (5% w/v) and incubated at 37°C for 48 h. Fifty-millilitre samples were withdrawn aseptically from each broth at 12, 24 and 48 h of

incubation and stored at 2°C. Cells were harvested by centrifugation (3000 x *g* for 15 min) using a Sorvall® RT7 refrigerated centrifuge (Newtown, CT, USA). The cell pellet (~1 mL) was washed twice in 20 mL of cold (2°C) 50 mM sodium citrate buffer (pH 5.5), centrifuging (3000 x *g* for 10 min) on both occasions (Scalabrini *et al.* 1998). The cells were then suspended in 10 mL of cold (2°C) 50 mM sodium citrate buffer (pH 5.5) and placed in an ice bath. To extract the enzyme, the cell suspension was homogenised according to the method of Smart *et al.* (1993). One gram of sterile glass beads of 0.25 to 0.30 mm diameter (B. Braun Melsungen AG, Melsungen, Germany) were added to the cell suspension at an approximate ratio of 1 part cells to 1 part glass beads. The cell suspension was then homogenised for 3 min using an MSK cell homogeniser (B. Braun Melsungen AG, Melsungen, Germany). Cell debris and glass beads were removed by centrifugation (10,000 x *g* for 30 min) using a Sorvall® RC28S refrigerated centrifuge. The supernatant was used for the analysis of β-glucosidase activity.

The β-glucosidase activity of the five *Bifidobacterium* strains was assayed by determining the rate of hydrolysis of the substrate p-nitrophenyl-β-D-glucopyranoside (pNPβG). Five-hundred micro litres of diluted enzyme extract was added to 1000 µL of 5 mM pNPβG, prepared in 100 mM sodium phosphate buffer (pH 7), and incubated at 37°C for 30 min. The reaction was terminated by adding 1000 µL of 1 M cold (4°C) sodium carbonate. The amount of p-nitrophenol released was measured with a spectrophotometer (Pharmacia LKB®, Novospec II™, Uppsala, Sweden) at 420 nm. One unit of enzyme was defined as the amount of enzyme that released 1 µmol of p-nitrophenol from the substrate pNPβG, per mL per min under assay conditions, as described by Scalabrini *et al.* (1998). Specific activity was expressed as units of enzyme per mg of protein. The protein concentration of the enzyme extract was determined by a modified version of the Lowry protein assay (Lowry *et al.* 1951) as described by Rosenberg (1996). p-Nitrophenol and bovine albumin (5 % w/v in 0.7 % w/v NaCl) were used as standards in the enzymic and protein assays, respectively. Concentrations of 0.5, 1, 1.5 and 2 µmol of p-nitrophenol and 10, 20, 40, 50 and 80 µg of bovine albumin were used to prepare standard

linear curves to quantify p-nitrophenol and protein. The pNPβG substrate, p-nitrophenol and bovine albumin were purchased from Sigma.

3.2.5 Fermentation of soymilk by bifidobacteria

The strains of *Bifidobacterium* showing detectable levels of β-glucosidase activity were used for the fermentation of soymilk. Strains were activated in MRS broth with additional 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine-HCl by 2 successive transfers. The third transfer was carried out in sterile soymilk (in triplicate). An inoculum level of 5% (v/v) was used for strain activation and incubation was at 37 °C for 20 h. For soymilk fermentation studies, 500 mL of sterile soymilk stored in glass bottles was inoculated (in triplicate) with active culture of *Bifidobacterium* sp. (5% v/v) and incubated at 37°C for 48 h. Samples of 100 mL were withdrawn aseptically at 0, 12, 24, 36 and 48 h of incubation for enumeration of *Bifidobacterium* populations and the remainder stored at –20°C. A fifty-millilitre aliquot was taken from each thawed sample and freeze-dried using a Dynavac® FD300 Freeze Drier (Rowville, Victoria, Australia) for the extraction of isoflavone and analysis using HPLC.

3.2.6 Enumeration of bifidobacteria in fermented soymilk

The pour plate method was used for the enumeration of viable populations of *Bifidobacterium*. MRS agar with additional 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine-HCl was used for enumeration and the plates were incubated at 37°C for 3 days in an anaerobic jar (Becton Dickinson Microbiology Systems®, Sparks, MD, USA) with a Gas Generating Kit™ (Oxoid Ltd.). Populations of bifidobacteria are presented as $\log_{10}$ colony forming units (CFU) per mL soymilk.

3.2.7 Extraction of isoflavones for HPLC analysis

The extraction of isoflavones, including malonyl-, acetyl- and β-glucosides, and aglycone isomers, from fermented and non-fermented soymilks, was performed in duplicate using a modified version of a method described by Setchell *et al.* (2001). One gram of freeze-dried soymilk was added to 50 mL of

80% (v/v) aqueous methanol in a 150 mL round bottom flask and refluxed on a heating mantle for 1 h. The mixture was then filtered through a Whatman 1 filter paper (Whatman International Ltd., Maidstone, Kent, UK) into a 100 mL volumetric flask. The remaining dried soy matter was washed with 20 to 30 mL of 80% (v/v) aqueous methanol and filtered into the same flask. A five-millilitre aliquot was taken and with the addition of 30 μL of equilenin solution (50 mg per 25mL) dried under a stream of nitrogen using a Pierce® model 18780 nine-needle evaporating unit (Pierce Biotechnology Inc., Rockford, Ill, USA). The resultant dried matter was then resuspended in 1 mL of 10 mM ammonium acetate buffer (containing 0.1% trifluoro-acetic acid) and acetonitrile (50:50) solution and centrifuged (3000 x *g* for 15 min) using a Hettich® Universal™ centrifuge (Tuttlingen, Germany) prior to transferring to HPLC vials.

3.2.8 Isoflavone standards

Aglycone standards of genistein, daidzein and glycitein (synthetic) were purchased from Sigma. Standards of genistin, daidzin and glycitin (β-glucoside isomers) were purchased from Indofine Chemical Company (Somerville, NJ, USA). Equilenin, used as an internal standard (ISTD), was supplied from Riedel deHaen™ (Castle Hill, NSW, Australia). Genistin, genistein, daidzein, and equilenin were prepared in HPLC grade methanol, and daidzin, glycitin and glycitein in ethanol, as they varied in solubility characteristics. Mixed and single isoflavone standards were dried under a stream of nitrogen using a Pierce® model 18780 nine-needle evaporating unit and then resuspended in 1 mL of 10 mM ammonium acetate buffer (containing 0.1% trifluoro-acetic acid) and acetonitrile (50:50) solution prior to injection onto the column.

3.2.9 Reversed-phase HPLC apparatus and reagents

Chromatographic analyses were carried out on a Hewlett Packard® 1100 series High Performance Liquid Chromatograph (Agilent Technologies, Forest Hill, Victoria, Australia) with auto sampler, quaternary pump, diode array ultraviolet-visible (UV/VIS) detector, vacuum degasser and

thermostatically controlled column compartment. A Keystone Scientific® (Bellefone, PA, USA) ODS-C18 (250 x 4.6 mm internal diameter; 5 μm) reversed-phase column was used to separate the isoflavone isomers. HPLC grade methanol and acetonitrile were purchased from Labscan Analytical Sciences (Bangkok, Thailand), and trifluoro-acetic acid, absolute ethanol and ammonium acetate from Sigma. All reagents used in the extraction of isoflavone and HPLC analyses were filtered through a 0.5 μm FH membrane (Millipore®, Bedford, MA, USA).

3.2.10 HPLC analysis of isoflavones

Gradient elution was used to isolate the isoflavones for detection and was composed of 90% (v/v) aqueous acetonitrile (solvent A) and 10 mM ammonium acetate buffer containing 0.1% trifluoro-acetic acid (solvent B), set at a flow rate of 1 mL per min (Setchell *et al.* 2001). After the 10 μL injection of sample or isoflavone standard onto the column (25°C), solvent B was set at 100 % for 2 min, reduced to 50% over 22 min, followed by 50% for 5 min, increased to 100% over 6 min, and finally 100% for 5 min prior to the next injection (Setchell *et al.* 2001). The diode array UV/VIS detector was set at a wavelength of 260 nm to detect malonyl-, acetyl- and β-glucosides, aglycones and equilenin (ISTD).

Four mixed standards containing all isoflavone β-glucoside and aglycone isomers at equal concentration (20, 50, 80 and 100 ng per 10 μL) were used for the quantification of isoflavone isomers; calculated back to wet basis (expressed as mg isoflavone per 100 mL of soymilk) (Appendix A). Concentrations of isoflavone were calculated according to the peak area response of equilenin (ISTD), which was added to each isoflavone mixed standard at a concentration of 600 ng per 10 μL (Appendix A). Retention times for aglycone and β-glucoside isoflavone isomers were determined using single standards and those of malonyl- and acetyl-glucoside isomers were based on the retention times reported by Setchell *et al.* (2001) under identical HPLC conditions. Malonyl- and acetyl-glucoside conjugates were quantified with respect to their β-glucoside equivalent response factors and corrected

according to molecular weight (Appendix B). Kudou *et al.* (1991) showed that the molar extinction coefficients of the malonyl-glucoside conjugates approximate those of β-glucoside conjugates.

3.2.11 Evaluating the precision of the HPLC method used to quantify isoflavones

Intra- and inter-assay percentage coefficients of variation (%COV) for the concentration of each isoflavone isomer in SPI590 powder were evaluated. Five replicates of SPI590 (1.00 g each) analysed alongside freeze dried soymilk on the same day were used to determine intra-assay %COV. Inter-assay %COV was determined by extracting and analysing isoflavones in one sample of SPI590 powder on consecutive days (n = 5) whilst conducting analyses on freeze-dried soymilk. Values of intra- and inter-assay %COV shown below represent the standard deviation divided by the mean (n = 5) multiplied by a factor of 100.

The intra- and inter-assay %COV for each isoflavone isomer concentration in SPI590 was as follows: daidzein, 7.6, 8.1; genistein, 2.6, 3.5; glycitein, 7.1, 21.0; daidzin, 3.8, 2.0; genistin, 9.0, 6.0; glycitin, 2.9, 2.2; malonyldaidzin, 31.7, 32.8; malonylgenistin, 4.0, 2.3; malonylglycitin, 13.2, 7.8; acetyldaidzin, 12.5, 11.6; acetylgenistin, 3.7, 3.8; and acetylglycitin, 18.6, 22.3, respectively. The intra-and inter-assay %COV for the total isoflavone concentration found in SPI590 was 5.8 and 3.5, respectively, in line with previous studies (intra- and inter-assay %COV of ≤ 5 and ≤ 10, respectively) (Wang *et al.* 1990; Coward *et al.* 1993; Barnes *et al.* 1994; Song *et al.* 1998; Murphy *et al.* 1999).

3.2.12 Statistical analysis

Growth of each *Bifidobacterium* strain for β-glucosidase assays and for the fermentation of soymilk was performed in triplicate on two occasions. Assays for β-glucosidase activity, enumeration of bifidobacteria, and quantification of isoflavones were performed in duplicate and are presented as a mean ± standard error of twelve replicates. To find significant differences in β-glucosidase activity between strains as well as differences in *Bifidobacterium* populations and isoflavone isomer

concentrations between soymilks, means were analysed with one-way analysis of variance (ANOVA) and 95% confidence intervals using Microsoft® Excel StatPro™ as described by Albright *et al.* (1999). ANOVA data with a $P<0.05$ was classified as statistically significant.

3.3 RESULTS AND DISCUSSION

3.3.1 β-Glucosidase activity of bifidobacteria

The β-glucosidase activity of five strains of *Bifidobacterium* are shown in Table 3.1. Four of the five strains of bifidobacteria showed detectable levels of β-glucosidase activity when grown in at least two of the four broths. Although the genus *Bifidobacterium* in general is reported to produce β-glucosidase, BI1912 did not exhibit any activity under the assay conditions. Tochikura *et al.* (1986) found that the β-glucosidase activity of twelve strains of *Bifidobacterium* propagated in glucose-supplemented broth varied significantly, with two strains showing no β-glucosidase activity even after overnight incubation. As shown in Table 3.1, there was a significant difference in β-glucosidase activity observed between BP20099, BB536, BL1941 and BB12 within each broth ($P<0.05$). The highest activity was observed in MRS-gluc broth, for BL1941. β-Glucosidase was highest at 12 or 24 h of incubation, which corresponds to the exponential phase of bacterial growth, and decreased during the stationary phase (> 24 h of incubation). These results are in agreement with those of Scalabrini *et al.* (1998) and Roy *et al.* (1991).

MRS broth is ideal for the growth of bifidobacteria due to the presence of glucose. Glucose is metabolised via the fructose-6-phosphate pathway with the help of fructose-6-phosphate phosphoketolase, the enzyme necessary for the growth of bifidobacteria (Scardovi & Trovatelli, 1965). As shown in Table 3.1, bifidobacteria grown in MRS-gluc broth produced higher levels of β-glucosidase overall, as compared with MRS, MRS-lac and MRS-raf broth. The glucose content of the MRS broth formulation (that is, 2% w/v) only supported the production of β-glucosidase (at detectable

levels) for two of the five strains of bifidobacteria. The presence of additional 1% (w/v) glucose in MRS-gluc broth enhanced the production of this enzyme, to detectable levels for strains BP20099 and BB536. Hence, MRS-gluc broth appeared to be the most effective medium for the growth of bifidobacteria for the production of β-glucosidase. The four strains showing enzymic activity in MRS-gluc broth possibly grew exponentially for an extended period due to an additional glucose level (that is, 3% w/v) and therefore produced the highest levels of β-glucosidase after 24 h of incubation. This was followed by successive stationary and death phases of bacterial growth with each strain consistently showing a reduction in β-glucosidase at 48 h of incubation.

Overall, BB12 was the highest producer of β-glucosidase in MRS-lac and MRS-raf broth when compared to the other strains (Table 3.1). Ballongue (1993) states that bifidobacteria produce a variety of intracellular saccharolytic enzymes utilised in breaking down di-, tri- and other oligosaccharides to simple hexose monomers required as a nutrient supply. Furthermore, Tochikura *et al.* (1986) reported that *Bifidobacterium* sp. produced an intracellular crude enzyme comprising β-glucosidase, β-galactosidase and α-galactosidase at varying levels, when grown in only glucose supplemented medium. BB12 was most β-glucosidase active in MRS broth supplemented with lactose; however, lactose is not found in soymilk (Liu, 1997). BL1941 did not produce β-glucosidase in the presence of any other sugar except glucose as fermentable substrate. Thus, BL1941 was not considered a promising strain for subsequent soymilk fermentation experiments, as soymilk is a heterogenous source of carbohydrates, including raffinose, stachyose and glucose (Liu, 1997). BP20099 and BB536 showed similar β-glucosidase activities when grown in MRS and MRS-lac broth ($P>0.05$). In contrast, BP20099 and BB536 produced significantly different levels of β-glucosidase at 12, 24 and 48 h of incubation in MRS-gluc broth ($P<0.05$), and at 12 h of incubation in MRS-raf broth ($P<0.05$).

3.3.2 Fermentation of soymilk by bifidobacteria

Viable populations of four *Bifidobacterium* strains in soymilk after 12, 24, 36 and 48 h of incubation at 37°C are shown in Table 3.2. The greatest exponential increases in the populations of *Bifidobacterium* occurred over the first 12 h of incubation. Growth reduced between 12 and 24 h of incubation, which may have reflected a change from exponential to stationary growth phases, followed by a decrease in bacterial numbers between 24 and 48 h of incubation. The low level of mono- and oligo-saccharides in soymilk made with SPI may have restricted the proliferation of these strains beyond 12 h of incubation, with the fall in bacterial growth between 12 and 24 h an indication of a diminishing nutrient supply. Liu (1997) stated that a fraction of soluble carbohydrates are lost with the removal of insoluble carbohydrates and lipids in the mild alkali extraction process used in the manufacture of SPI. In comparison, Kamaly (1997) found that *B. longum* and *B. bifidum* grew effectively in plain soymilk made of soybeans over 24 h of incubation at 37 °C, with population increases of 1.8 and 2.4 $\log_{10}$ CFU per mL, respectively. The predominant α-galactosyl oligosaccharide content of soymilk made of soybeans, including raffinose and stachyose, was found by Pinthong *et al.* (1980) as a satisfactory fermentable substrate for the growth of bifidobacteria. Furthermore, Scalabrini *et al.* (1998) found that *Bifidobacterium* sp. produced intracellular α-galactosidase, which enabled this genus to reduce the content of stachyose and raffinose in soymilk made of soybeans after 24 and 48 h of fermentation to at least half their original concentration or to undetectable levels.

Strains of BP20099 and BB536 displayed the best growth characteristics in soymilk (Table 3.2). Over the first 12 h of incubation, BP20099 and BB536 exhibited significantly greater increases in viable population (1.33 and 1.32 $\log_{10}$ CFU per mL, respectively) compared to BB12 and BL1941 ($P<0.05$). The viable count of BB12 increased from 7.39 (at 0 h) to 8.17 (at 12 h) $\log_{10}$ CFU per mL during exponential growth, a difference of 0.78 $\log_{10}$ CFU per mL. The high β-glucosidase activity of BB12 in MRS-raf broth (Table 3.1) did not appear to stimulate its growth in soymilk, possibly due to the predominant raffinose and stachyose concentration of soymilk which requires α-galactosidase for

metabolism (Scalabrini *et al.* 1998). BL1941 showed the poorest growth characteristics in soymilk, with only a 0.13 $\log_{10}$ CFU per mL increase during the first 12 h of incubation. There was a reduction in the viable count of BL1941 after only 12 h of incubation. Both the β-glucosidase assays and soymilk fermentation experiments indicated that BL1941 was unable to proliferate in a medium containing a mixture of mono- and oligo-saccharides.

3.3.3 HPLC analysis of isoflavones in soymilk

The approximate retention times of malonyl-, acetyl-, β-glucoside and aglycone isoflavone isomers found in soymilk fermented by *Bifidobacterium* are shown in Figure 3.1. In line with previous work by Setchell *et al.* (2001), malonylglucoside isoflavones eluted first, followed in order by the isoflavone structures of β-glucoside, acetylglucoside and aglycone. The isoflavone isomers were eluted according to their polarity and hydrophobic interaction with the reversed-phase HPLC column. Hence, the malonylglucosides of the highest polarity eluted first followed by less polar compounds, with respect to their chemical structure and number of hydroxyl groups (Figure 3.2). In the study of Coward *et al.* (1998) and King & Bignell (2000), β-glucosidic isoflavone isomers eluted first, followed in order by malonylglucosides, acetylglucosides and aglycones when using a mobile phase of acetonitrile and 0.1% trifluoro-acetic acid. Amongst each chemical form of isoflavone, daidzein consistently eluted first followed by glycitein and genistein (Figure 3.1), in agreement with Coward *et al.* (1998) and King & Bignell (2000). The chosen internal standard, equilenin, eluted at 29 min, separated from the isoflavone isomers to prevent overlapping (Figure 3.1).

3.3.4 Concentration of isoflavone in non-fermented soymilk

The concentrations of isoflavone isomers found in non-fermented soymilk (that is, 0 h) are shown in Tables 3.3 to 3.6. Non-fermented soymilk made from SPI590 (4% total solids) contained a total of 4.956 mg isoflavones per 100 mL. The non-bioavailable β-glucoside forms contributed the greatest concentration of isoflavone (83%), with a total of 4.111 mg per 100 mL ($P<0.05$). The concentration of

genistin was the highest of individual isomers, at 2.674 mg per 100 mL ($P<0.05$). Only 8% of total isoflavones were present in a bioactive isoflavone aglycone configuration. King & Bignell (2000) also found that the β-glucoside forms comprised greater than 80% of the total isoflavone concentration in soymilk, followed by the aglycones and malonyl- and acetyl-glucoside conjugates.

3.3.5 Enzymic transformation of isoflavone isomers in soymilk fermented by bifidobacteria

Figure 3.2 displays the structural transformation of isoflavones caused by enzymic hydrolysis and organic reduction that occurred in soymilk fermented by *Bifidobacterium*. As a result, changes in the concentration of malonyl-, acetyl-, β–glucoside and aglycone isoflavone isomers occurred in soymilks fermented by BP20099, BB536, BL1941 and BB12, shown in Tables 3.3, 3.4, 3.5 and 3.6, respectively. Of the four strains grown in soymilk, BP20099, BB536 and BB12 caused a significant increase in isoflavone aglycone concentrations ($P<0.05$), potentially via the β–glucosidase-catalysed hydrolysis of isoflavone glucoside conjugates. In parallel, the concentrations of isoflavone malonyl-, acetyl- and β-glucosides were significantly reduced by BP20099, BB536 and BB12 ($P<0.05$).

Changes in the concentration of individual isoflavone isomers (malonyl-, acetyl-, β–glucoside and aglycone forms) occurring in soymilks fermented by BP20099, BB536, BL1941 and BB12 are also shown in Tables 3.3, 3.4, 3.5 and 3.6, respectively. The concentrations of individual aglycone structures, including daidzein, glycitein and genistein, increased significantly over the initial 24 h exponential growth phase of BP20099, BB536 and BB12 ($P<0.05$), followed by a reduced level of enzymic transformation into isoflavone aglycone between 24 and 48 h of incubation ($P>0.05$). Genistein contributed the greatest concentration of bioactive isoflavone aglycone in each of the soymilks fermented by BP20099, BB536 and BB12. This was due possibly to the higher concentration of genistein and genistin in non-fermented soymilk compared to the other isomers and the significant transformation of genistin into genistein during fermentation ($P<0.05$). In soymilk fermented by BB12, there was a 9.5 fold increase in the concentration of genistein after 24 h of incubation, with some 90%

of the original 2.674 mg of genistin per 100 mL transformed into genistein. In comparison, the concentration of daidzein and glycitein only increased a maximum of 4.7 and 3.8 fold, respectively, in soymilk fermented by BB12 after 24 h of incubation. In this case, 85% of the concentration of daidzin (at 1.062 mg per 100 mL) and 39% of the concentration of glycitin (at 0.375 mg per 100 mL) found in non-fermented soymilk was transformed into their respective aglycone isomers during the first 24 h of incubation of BB12. Wang & Murphy (1996) found that growth of fungi *Rhizopus oligosporus* in cooked soybeans in the manufacture of tempeh caused a 7.3 and 6.2 fold increase in the concentration of daidzein and genistein, respectively, after 22 h of incubation at 37 °C ($P<0.05$). They also reported a significant decrease in the concentration of daidzin (from 16.8 to 2.5 mg per 100 g) and genistin (from 32.2 to 11.6 mg per 100 g) after fermentation ($P<0.05$) (Wang & Murphy, 1996). This was possibly the result of fungal enzymatic hydrolysis of isoflavone glucosides to form aglycones, similar to that occurring in soymilk fermented with *Bifidobacterium*.

Varying levels of isoflavone glucoside hydrolysis (with the increase in aglycone concentrations) occurred between soymilks fermented by BP20099, BB536, BL1941 and BB12. As shown in Table 3.3, soymilk fermented by BP20099 showed a significant increase in aglycone concentration from 0.381 mg (at 0 h) to 1.892 mg per 100 mL after 24 h of incubation ($P<0.05$), with β-glucoside isoflavone structures decreasing from 4.111 mg to 1.808 mg per 100 mL over the same period ($P<0.05$). The concentration of aglycone formed in soymilk fermented by BB536 exceeded that of BP20099 after 24 h of incubation, at 2.083 mg per 100 mL (Table 3.4). However, the greatest increase in the concentration of bioactive isoflavone aglycone occurred in soymilk fermented by BB12, from 0.381 mg (at 0 h) to 2.704 mg per 100 mL after 24 h of incubation (Table 3.6). Of the isoflavone glucosides, the β-glucosidic forms appeared to undergo the most biotransformation into aglycone structures, especially in the presence of BB12; reducing from 4.111 mg to 0.661 mg per 100 mL ($P<0.05$) (Table 3.6). In contrast, there was no significant enzymic transformation of isoflavone glucosides into bioactive aglycones in soymilk fermented by BL1941 ($P<0.05$) (Table 3.5). Very little change in the

concentration of malonyl- and β-glucoside isomers was observed in soymilk fermented by BL1941 after 48 h of incubation ($P>0.05$) (Table 3.5), possibly due to poor production of β-glucosidase. BL1941 produced undetectable levels of β-glucosidase when grown in MRS broth containing additional raffinose (Table 3.1); raffinose is found in soymilk (Pinthong *et al.* 1980). However, acetylglucoside isomers were significantly hydrolysed by BL1941 ($P<0.05$), but, because of the low content of acetylglucosides in the non-fermented soymilk there was no major increase in aglycone isomer concentration ($P>0.05$) (Table 3.5).

A mass balance of the total concentration of isoflavones in soymilk before and after 12, 24, 36 and 48 h of incubation with BP20099, BB536, BL1941 and BB12 are shown in Tables 3.3, 3.4, 3.5 and 3.6, respectively. The most significant losses in total isoflavone concentration occurred in the first 12 h of incubation in soymilks fermented by BP20099, BB536 and BB12 ($P<0.05$). This corresponded to the exponential growth phase of each of these strains in soymilk. Significant losses in total isoflavone concentration ($P<0.05$) only occurred in soymilks where there were significant decreases in the concentration of isoflavone glucosides caused by enzymic hydrolysis ($P<0.05$); that is, in soymilks fermented by BP20099, BB536 and BB12. Soymilk fermented by BB12, which showed the most significant transformation of isoflavone glucosides to aglycones ($P<0.05$), also depicted the greatest losses in total isoflavone concentration, from 4.956 mg (at 0 h) to 4.026 mg per 100 mL after 12 h of incubation (Table 3.6). Losses in total isoflavone concentration after fermentation were possibly caused by the hydrolytic cleavage of the glucose moiety from the isoflavone glucoside isomers, which contribute to the mass of isoflavones when found as glucoside forms (King & Bignell, 2000).

There appeared to be correlations between the β-glucosidase activity of each *Bifidobacterium* strain, their growth in soymilk, and the biotransformation of isoflavone glucosides into bioactive forms. For example, soymilk fermented by BB12 contained the highest concentration of aglycones after 24 h of incubation (Table 3.6) and this strain also showed the highest β-glucosidase activity in MRS broth

containing raffinose (Table 3.1). BP20099 and BB536, which showed significantly lower levels of β-glucosidase activity in MRS broth containing raffinose (Table 3.1), were not as effective as BB12 in hydrolysing isoflavone glucosides into aglycone forms during soymilk fermentation. In the case of BL1941, which showed undetectable levels of β-glucosidase activity in MRS broth containing raffinose (Table 3.1) was reflected in its inability to produce the enzyme in soymilk, with poor biotransformation of isoflavone glucosides (Table 3.5). Finally, the poor growth of BB12 in soymilk in comparison to BP20099 and BB536 (Table 3.2) did not affect its ability to produce intracellular β-glucosidase and hydrolyse isoflavone glucosides.

3.4 CONCLUSIONS

Strains of BP20099, BB536, BL1941 and BB12 produced varying levels of β-glucosidase depending on the sugar content of MRS broth and stage of incubation. Of the four strains that produced β-glucosidase, BP20099, BB536 and BB12 were capable of growing in soymilk. These three strains also transformed the predominant isoflavone malonyl-, acetyl- and β-glucosides found in soymilk to bioactive aglycones. The level of aglycones increased from 8% in non-fermented soymilk to approximately 50% due to fermentation by bifidobacteria. The reduction in the concentration of malonyl-, acetyl- and β-glucoside isomers varied considerably, with approximately 50%, 65% and 88% hydrolysed in soymilk fermented by BB12, respectively. In addition to the benefits conferred by bifidobacteria in relation to the intestinal health of humans, these bacteria may also alter the biological activity of soymilk by transforming the predominant concentration of isoflavone glucosides into aglycones.

Table 3.1 β-Glucosidase activity (units of enzyme per mg protein[1]) of *Bifidobacterium* sp. incubated for 12, 24 and 48 h at 37°C (mean ± standard error; n = 12)

Growth Medium	Incubation Time (h)	BP20099	BB536	BL1941	BB12	BI1912
MRS broth	12	$<0.0001^{cd}$	$<0.0001^{d}$	0.714 ± 0.006^{a}	0.534 ± 0.005^{b}	ND
	24	$<0.0001^{cd}$	$<0.0001^{d}$	0.779 ± 0.004^{a}	0.440 ± 0.003^{b}	ND
	48	$<0.0001^{cd}$	$<0.0001^{d}$	0.452 ± 0.002^{a}	0.162 ± 0.001^{b}	ND
MRS-gluc broth	12	0.846 ± 0.005^{c}	0.516 ± 0.013^{d}	3.892 ± 0.031^{a}	2.170 ± 0.012^{b}	ND
	24	0.937 ± 0.016^{c}	0.572 ± 0.005^{d}	4.625 ± 0.034^{a}	2.470 ± 0.008^{b}	ND
	48	0.505 ± 0.003^{c}	0.352 ± 0.006^{d}	3.945 ± 0.029^{a}	1.865 ± 0.012^{b}	ND
MRS-lac broth	12	0.018 ± 0.001^{bc}	$<0.0001^{c}$	ND	3.651 ± 0.151^{a}	ND
	24	0.249 ± 0.002^{bc}	$<0.0001^{c}$	ND	3.047 ± 0.215^{a}	ND
	48	$<0.0001^{bc}$	$<0.0001^{c}$	ND	2.081 ± 0.027^{a}	ND
MRS-raf broth	12	0.102 ± 0.002^{b}	0.040 ± 0.002^{c}	ND	0.474 ± 0.003^{a}	ND
	24	0.006 ± 0.002^{c}	0.042 ± 0.002^{bc}	ND	0.780 ± 0.020^{a}	ND
	48	$<0.0001^{bc}$	$<0.0001^{c}$	ND	0.591 ± 0.012^{a}	ND

BP20099: *B. pseudolongum*; BB536: *B. longum*; BL1941: *B. longum*; BB12: *B. animalis*; BI1941: *B. infantis*.

MRS-gluc: MRS broth containing additional 1% (w/v) D-glucose; MRS-lac: MRS broth containing additional 1% (w/v) lactose; MRS-raf: MRS broth containing additional 1% (w/v) raffinose.

ND: No activity detected in cell-free enzyme extract after 30 min of incubation.

<0.0001: Activity of less than 0.0001 U per mg protein found in cell-free enzyme extract after 30 min of incubation.

Means in the same row with different superscript are significantly different ($P<0.05$).

[1]One unit of enzyme was defined as the amount of enzyme that released 1 μmol of p-nitrophenol from pNPβG per mL per min under assay conditions.

Table 3.2 Viable populations of *Bifidobacterium* sp. in soymilk (log_{10} CFU per mL) incubated for 12, 24, 36 and 48 h at 37°C (mean ± standard error; n = 12)

Bifidobacteria	**Log_{10} CFU per mL change between intervals[1] (n = 12)**								
Strains	**0 h**		**12 h**		**24 h**		**36 h**		**48 h**
BP20099	6.60±0.03	+1.33^{a}	7.93±0.15	+0.19^{ab}	8.12±0.06	−0.10^{a}	8.02 ± 0.06	−0.05^{ab}	7.97 ± 0.07
BB536	6.69±0.05	+1.32^{a}	8.01±0.11	+0.27^{a}	8.28±0.03	−0.06^{a}	8.22 ± 0.02	−0.03^{a}	8.19 ± 0.03
BL1941	6.56±0.03	+0.13^{c}	6.69±0.03	−0.15^{b}	6.54±0.03	−0.33^{b}	6.21 ± 0.06	−0.11^{b}	6.10 ± 0.07
BB12	7.39±0.05	+0.78^{b}	8.17±0.03	+0.16^{ab}	8.33±0.06	−0.08^{a}	8.25 ± 0.09	−0.04^{ab}	8.21 ± 0.10

BP20099: *B. pseudolongum*; BB536: *B. longum*; BL1941: *B. longum*; BB12: *B. animalis*.
Means in the same column with different superscript are significantly different ($P<0.05$).
[1]Mean of the difference in log_{10} CFU per mL between incubation time intervals.

Table 3.3 Concentration of isoflavone isomers in soymilk (mg per 100 mL) fermented by BP20099 for 12, 24, 36 and 48 h of incubation at 37°C (mean ± standard error; n = 12)

Isoflavone Isomer	Incubation Time				
	0 h	12 h	24 h	36 h	48 h
Daidzein	0.120±0.009^{d}	0.282±0.010^{c}	0.433±0.008^{b}	0.465±0.006^{ab}	0.485±0.012^{a}
Genistein	0.202±0.021^{d}	0.785±0.031^{c}	1.316±0.024^{b}	1.466±0.024^{a}	1.570±0.054^{a}
Glycitein	0.059±0.005^{d}	0.093±0.004^{c}	0.144±0.004^{b}	0.162±0.003^{ab}	0.172±0.006^{a}
Aglycones[1]	0.381±0.035^{d}	1.159±0.043^{c}	1.892±0.034^{b}	2.092±0.032^{ab}	2.226±0.072^{a}
Daidzin	1.062±0.019^{a}	0.658±0.031^{b}	0.377±0.017^{de}	0.392±0.014^{cde}	0.357±0.011^{e}
Genistin	2.674±0.043^{a}	1.849±0.076^{b}	1.152±0.049^{cde}	1.053±0.034^{de}	0.956±0.037^{e}
Glycitin	0.375±0.006^{a}	0.327±0.009^{b}	0.270±0.005^{c}	0.293±0.003^{cde}	0.293±0.008^{de}
β-Glucosides[1]	4.111±0.067^{a}	2.834±0.115^{b}	1.808±0.071^{cde}	1.738±0.051^{de}	1.596±0.056^{e}
Malonyldaidzin	0.013±0.001^{a}	0.015±0.001^{a}	0.008±0.002^{a}	0.008±0.002^{a}	0.009±0.002^{a}
Malonylgenistin	0.045±0.002^{a}	0.034±0.003^{b}	0.020±0.002^{cde}	0.019±0.003^{de}	0.019±0.002^{e}
Malonylglycitin	0.204±0.0002^{ab}	0.022±0.001^{a}	0.021±0.001^{ab}	0.019±0.001^{b}	0.020±0.001^{ab}
Malonylglucosides[1]	0.078±0.002^{a}	0.071±0.004^{a}	0.048±0.005^{bcd}	0.046±0.005^{d}	0.048±0.006^{cd}
Acetyldaidzin	0.108±0.004^{a}	0.054±0.004^{cd}	0.043±0.006^{d}	0.081±0.004^{b}	0.093±0.004^{ab}
Acetylgenistin	0.233±0.006^{a}	0.118±0.007^{b}	0.061±0.005^{c}	0.025±0.002^{de}	0.021±0.002^{e}
Acetylglycitin	0.044±0.003^{bcd}	0.056±0.011^{abcd}	0.079±0.012^{a}	0.043±0.001^{cd}	0.037±0.001^{d}
Acetylglucosides[1]	0.386±0.013^{a}	0.228±0.013^{b}	0.183±0.011^{cde}	0.149±0.004^{e}	0.151±0.006^{de}
Total isoflavones[2]	4.956±0.072^{a}	4.292±0.091^{bcde}	3.931±0.096^{e}	4.025±0.091^{cde}	4.021±0.055^{de}

BP20099: *B. pseudolongum*.

ND: Not detected in 1 g of freeze dried soymilk used to extract isoflavones with a sample injection volume of 10 μL.

Means in the same row with different superscript are significantly different ($P<0.05$).

[1]Mean total of three respective isomers.

[2]Mean total of malonyl-, acetyl-, β-glucoside, and aglycone isomers.

Table 3.4 Concentration of isoflavone isomers in soymilk (mg per 100 mL) fermented by BB536 for 12, 24, 36 and 48 h of incubation at 37°C (mean ± standard error; n = 12)

Isoflavone Isomer	Incubation Time				
	0 h	12 h	24 h	36 h	48 h
Daidzein	0.120±0.009^{d}	0.261±0.030^{c}	0.471±0.016^{b}	0.605±0.017^{a}	0.599±0.012^{a}
Genistein	0.202±0.021^{d}	0.727±0.094^{c}	1.448±0.052^{b}	1.973±0.076^{a}	1.986±0.060^{a}
Glycitein	0.059±0.005^{d}	0.089±0.008^{cd}	0.164±0.008^{b}	0.315±0.023^{a}	0.358±0.020^{a}
Aglycones[1]	0.381±0.035^{d}	1.077±0.132^{c}	2.083±0.075^{b}	2.893±0.113^{a}	2.943±0.092^{a}
Daidzin	1.062±0.019^{a}	0.670±0.056^{b}	0.339±0.018^{c}	0.123±0.017^{de}	0.083±0.002^{e}
Genistin	2.674±0.043^{a}	1.814±0.119^{b}	1.046±0.055^{c}	0.343±0.062^{de}	0.187±0.014^{e}
Glycitin	0.375±0.006^{a}	0.314±0.007^{bc}	0.270±0.009^{c}	0.155±0.019^{d}	0.092±0.010^{e}
β-Glucosides[1]	4.111±0.067^{a}	2.799±0.180^{b}	1.664±0.079^{c}	0.621±0.097^{de}	0.361±0.024^{e}
Malonyldaidzin	0.013±0.001^{a}	0.013±0.001^{a}	0.010±0.002^{a}	0.005±0.003^{a}	0.001±0.004^{a}
Malonylgenistin	0.045±0.002^{a}	0.033±0.003^{b}	0.021±0.002^{c}	0.012±0.001^{de}	0.011±0.001^{e}
Malonylglycitin	0.020±0.0002^{a}	0.022±0.0003^{a}	0.022±0.001^{a}	0.021±0.001^{a}	0.023±0.001^{a}
Malonylglucosides[1]	0.078±0.002^{a}	0.067±0.004^{ab}	0.052±0.006^{bcd}	0.038±0.003^{d}	0.043±0.004^{cd}
Acetyldaidzin	0.108±0.004^{a}	0.055±0.005^{a}	0.057±0.005^{a}	0.077±0.004^{a}	0.054±0.007^{a}
Acetylgenistin	0.233±0.006^{a}	0.091±0.008^{b}	0.044±0.005^{c}	0.013±0.001^{de}	0.009±0.001^{e}
Acetylglycitin	0.044±0.003^{bcd}	0.062±0.012^{abcd}	0.083±0.013^{a}	0.041±0.002^{cd}	0.035±0.001^{d}
Acetylglucosides[1]	0.386±0.013^{a}	0.209±0.015^{bcde}	0.184±0.015^{cde}	0.130±0.005^{e}	0.097±0.007^{de}
Total isoflavones[2]	4.956±0.072^{a}	4.152±0.056^{bcd}	3.983±0.144^{cd}	3.682±0.135^{de}	3.444±0.102^{e}

BB536: *B. longum*.

ND: Not detected in 1 g of freeze dried soymilk used to extract isoflavones with a sample injection volume of 10 μL.

Means in the same row with different superscript are significantly different ($P<0.05$).

[1]Mean total of three respective isomers.

[2]Mean total of malonyl-, acetyl-, β-glucoside, and aglycone isomers.

Table 3.5 Concentration of isoflavone isomers in soymilk (mg per 100 mL) fermented by BL1941 for 12, 24, 36 and 48 h of incubation at 37°C (mean ± standard error; n = 12)

Isoflavone Isomer	Incubation Time				
	0 h	12 h	24 h	36 h	48 h
Daidzein	0.120±0.009^{a}	0.109±0.008^{a}	0.107±0.009^{a}	0.112±0.006^{a}	0.127±0.011^{a}
Genistein	0.202±0.021^{a}	0.205±0.021^{a}	0.193±0.026^{a}	0.187±0.025^{a}	0.224±0.031^{a}
Glycitein	0.059±0.005^{a}	0.054±0.005^{a}	0.053±0.006^{a}	0.054±0.005^{a}	0.061±0.007^{a}
Aglycones[1]	0.381±0.035^{a}	0.368±0.034^{a}	0.353±0.040^{a}	0.353±0.036^{a}	0.412±0.049^{a}
Daidzin	1.062±0.019^{a}	1.048±0.016^{a}	1.005±0.017^{a}	0.994±0.014^{a}	1.096±0.056^{a}
Genistin	2.674±0.043^{a}	2.661±0.033^{a}	2.570±0.058^{a}	2.517±0.050^{a}	2.791±0.159^{a}
Glycitin	0.375±0.006^{a}	0.368±0.005^{a}	0.359±0.009^{a}	0.350±0.008^{a}	0.385±0.023^{a}
β-Glucosides[1]	4.111±0.067^{a}	4.077±0.050^{a}	3.934±0.083^{a}	3.861±0.071^{a}	4.273±0.237^{a}
Malonyldaidzin	0.013±0.001^{ab}	0.015±0.0003^{a}	0.013±0.001^{ab}	0.012±0.001^{b}	0.014±0.001^{ab}
Malonylgenistin	0.045±0.002^{a}	0.046±0.002^{a}	0.044±0.003^{a}	0.043±0.003^{a}	0.048±0.005^{a}
Malonylglycitin	0.020±0.0002^{ab}	0.023±0.0002^{a}	0.022±0.001^{ab}	0.018±0.001^{b}	0.021±0.002^{ab}
Malonylglucosides[1]	0.078±0.002^{a}	0.084±0.002^{a}	0.079±0.004^{a}	0.073±0.004^{a}	0.084±0.007^{a}
Acetyldaidzin	0.108±0.004^{a}	0.076±0.003^{de}	0.082±0.002^{bcde}	0.078±0.003^{cde}	0.074±0.008^{e}
Acetylgenistin	0.233±0.006^{a}	0.142±0.002^{b}	0.102±0.003^{c}	0.063±0.002^{de}	0.060±0.003^{e}
Acetylglycitin	0.044±0.003^{cde}	0.034±0.002^{e}	0.085±0.015^{a}	0.036±0.001^{de}	0.046±0.007^{bcde}
Acetylglucosides[1]	0.386±0.013^{a}	0.252±0.004^{c}	0.269±0.019^{bc}	0.177±0.004^{e}	0.180±0.017^{de}
Total isoflavones[2]	4.956±0.072^{a}	4.781±0.061^{a}	4.635±0.142^{a}	4.464±0.112^{a}	4.949±0.305^{a}

BL1941: *B. longum*.

ND: Not detected in 1 g of freeze dried soymilk used to extract isoflavones with a sample injection volume of 10 μL.

Means in the same row with different superscript are significantly different ($P<0.05$).

[1]Mean total of three respective isomers.

[2]Mean total of malonyl-, acetyl-, β-glucoside, and aglycone isomers.

Table 3.6 Concentration of isoflavone isomers in soymilk (mg per 100 mL) fermented by BB12 for 12, 24, 36 and 48 h of incubation at 37°C (mean ± standard error; n = 12)

Isoflavone Isomer	Incubation Time				
	0 h	12 h	24 h	36 h	48 h
Daidzein	0.120±0.009^{c}	0.465±0.008^{b}	0.564±0.005^{a}	0.584±0.009^{a}	0.598±0.019^{a}
Genistein	0.202±0.021^{c}	1.631±0.029^{b}	1.913±0.036^{a}	1.941±0.037^{a}	2.003±0.083^{a}
Glycitein	0.059±0.005^{d}	0.160±0.004^{c}	0.226±0.002^{b}	0.300±0.006^{a}	0.320±0.009^{a}
Aglycones[1]	0.381±0.035^{c}	2.256±0.040^{b}	2.704±0.041^{a}	2.825±0.048^{a}	2.921±0.110^{a}
Daidzin	1.062±0.019^{a}	0.263±0.015^{b}	0.155±0.003^{c}	0.093±0.002^{de}	0.085±0.002^{e}
Genistin	2.674±0.043^{a}	0.530±0.051^{b}	0.279±0.012^{cde}	0.182±0.005^{e}	0.209±0.003^{de}
Glycitin	0.375±0.006^{a}	0.257±0.008^{bc}	0.227±0.006^{c}	0.149±0.005^{de}	0.135±0.009^{e}
β-Glucosides[1]	4.111±0.067^{a}	1.050±0.074^{b}	0.661±0.021^{c}	0.424±0.009^{e}	0.429±0.011^{de}
Malonyldaidzin	0.013±0.001^{ab}	0.017±0.001^{a}	0.010±0.002^{ab}	0.006±0.002^{b}	0.010±0.003^{ab}
Malonylgenistin	0.045±0.002^{a}	0.010±0.001^{bcde}	0.010±0.0002^{cde}	0.010±0.001^{de}	0.010±0.0003^{e}
Malonylglycitin	0.020±0.0002^{a}	0.022±0.001^{a}	0.021±0.001^{a}	0.019±0.001^{a}	0.022±0.001^{a}
Malonylglucosides[1]	0.078±0.002^{a}	0.048±0.002^{bcd}	0.041±0.004^{de}	0.035±0.002^{e}	0.042±0.004^{cde}
Acetyldaidzin	0.108±0.004^{a}	0.068±0.002^{cd}	0.074±0.007^{bcd}	0.106±0.002^{a}	0.058±0.008^{d}
Acetylgenistin	0.233±0.006^{a}	0.044±0.004^{b}	0.017±0.001^{cd}	0.003±0.001^{de}	0.0003±0.0002^{e}
Acetylglycitin	0.044±0.003^{ab}	0.075±0.011^{ab}	0.067±0.009^{ab}	0.036±0.001^{b}	0.046±0.008^{ab}
Acetylglucosides[1]	0.386±0.013^{a}	0.186±0.014^{bcd}	0.154±0.004^{cd}	0.145±0.004^{de}	0.104±0.008^{e}
Total isoflavones[2]	4.956±0.072^{a}	3.540±0.140^{cde}	3.560±0.122^{bcde}	3.429±0.154^{e}	3.496±0.114^{de}

BB12: *B. animalis*.

ND: Not detected in 1 g of freeze dried soymilk used to extract isoflavones with a sample injection volume of 10 μL.

Means in the same row with different superscript are significantly different ($P<0.05$).

[1]Mean total of three respective isomers.

[2]Mean total of malonyl-, acetyl-, β-glucoside, and aglycone isomers.

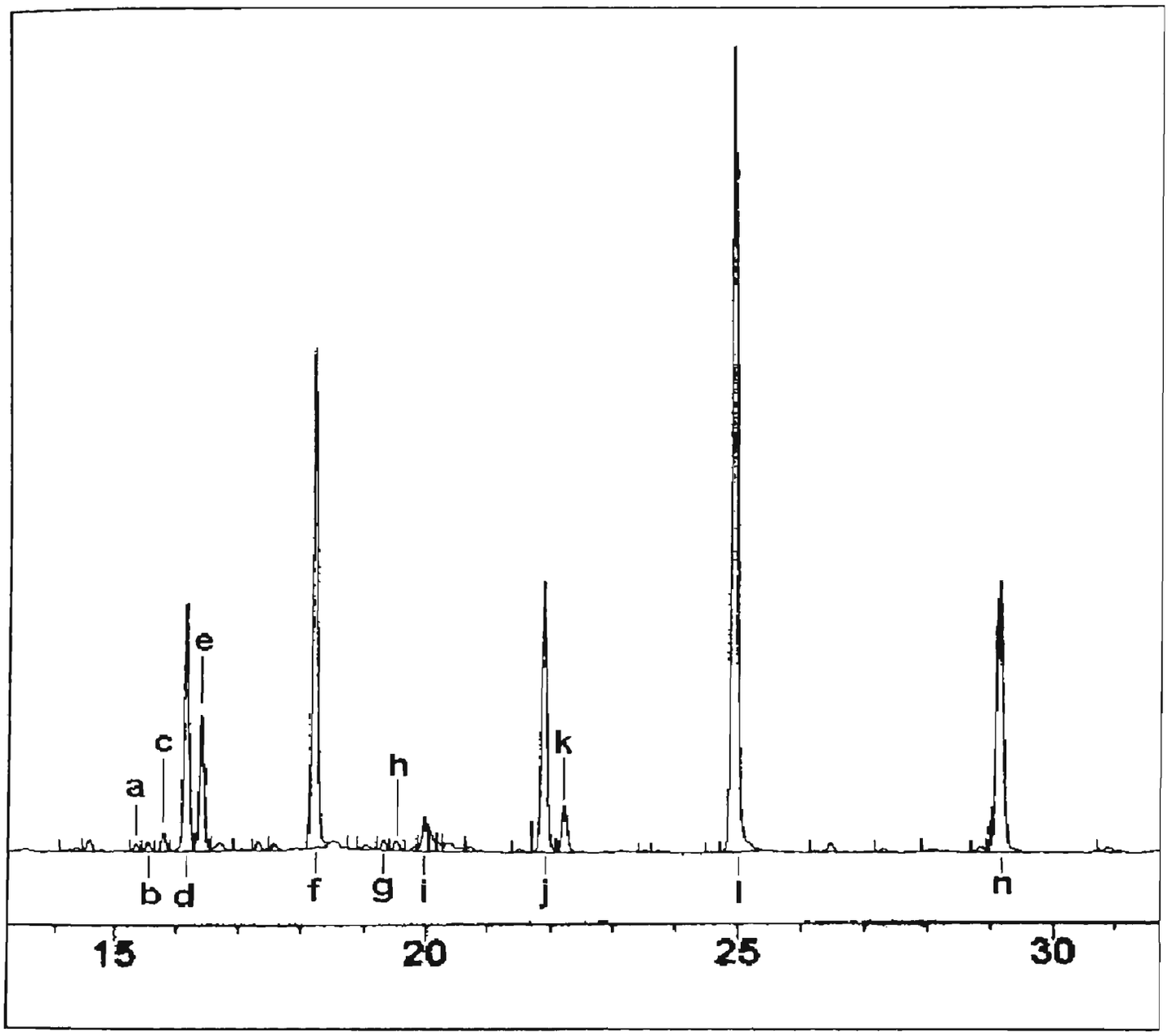

Figure 3.1 HPLC chromatogram showing the approximate retention times of isoflavone isomers (at wavelength of 260 nm) found in soymilk fermented by bifidobacteria; (a) malonyldaidzin, 15.34 min; (b) malonylglycitin, 15.55 min; (c) malonylgenistin, 15.79 min; (d) daidzin, 16.16 min; (e) glycitin, 16.41 min; (f) genistin, 18.21 min; (g) acetyldaidzin, 19.20 min; (h) acetylglycitin, 19.47 min; (i) acetylgenistin, 19.98 min; (j) daidzein, 21.88 min; (k) glycitein, 22.21 min; (l) genistein, 24.92 min; (n) equilenin internal standard, 29.12 min.

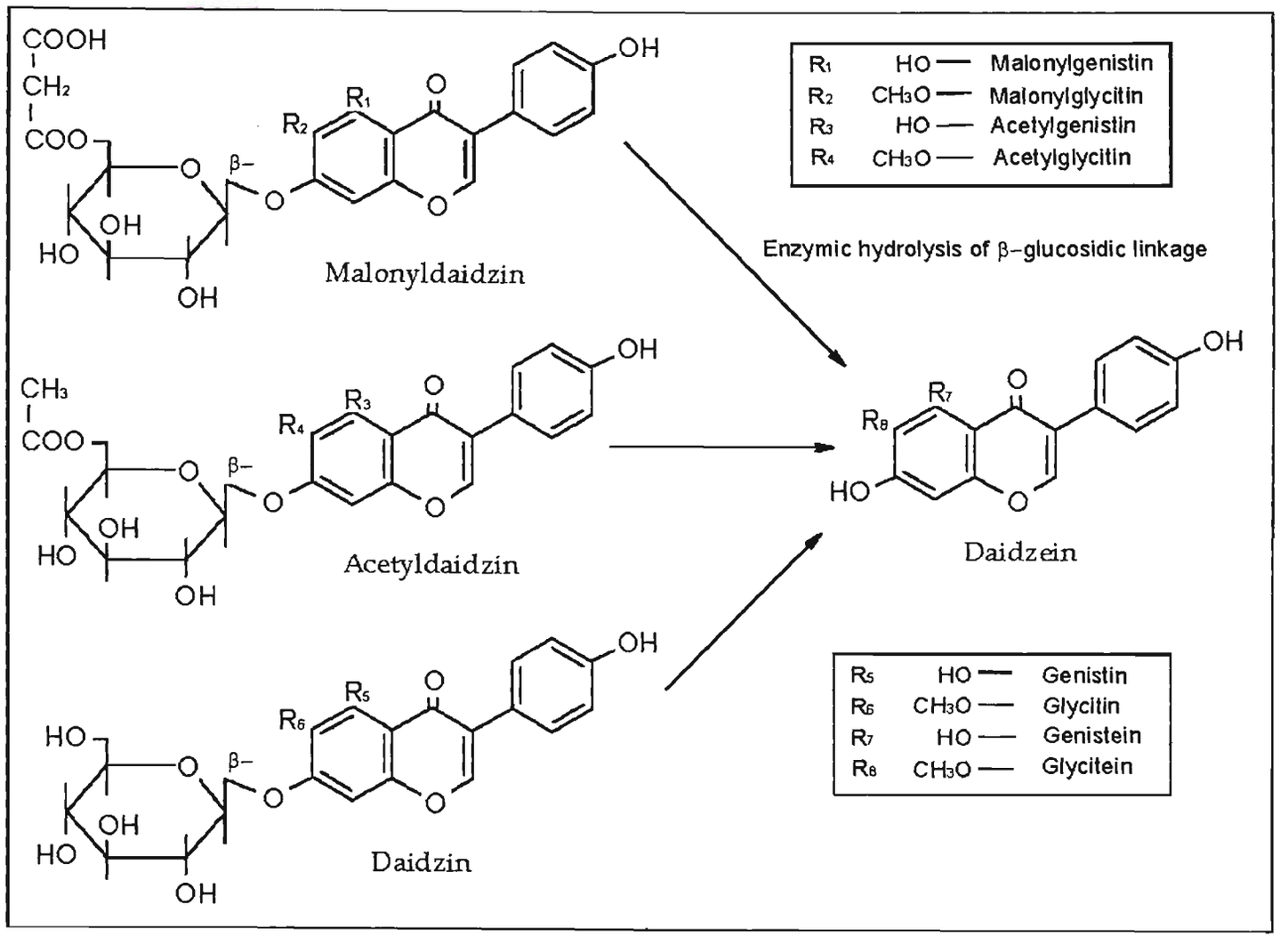

Figure 3.2 Structural representation of isoflavone isomers and the transformations caused by bacterial-induced enzymic hydrolysis.

4.0 Biotransformation of Isoflavones by Bifidobacteria in Fermented Soymilk Supplemented with D-Glucose and L-Cysteine

4.1 INTRODUCTION

Soymilk is potentially a nutritious growth medium for bifidobacteria due to its predominant α-galactosyl oligosaccharide (raffinose and stachyose) content. However, Kamaly (1997) found that the viable populations of *B. longum* and *B. bifidum* grown in soymilk (made from soybeans) were lower than those grown in skim milk and MRS broth (de Mann *et al.* 1960) after 24 h of incubation. This may be due to the lack of free glucose available in soymilk, as glucose is only a minor constituent of soybeans (Liu, 1997). Chou & Hou (2000) reported that supplementing soymilk made from soybeans with glucose significantly increased the growth of *B. infantis* over a 24 h incubation period ($P<0.05$) when compared to plain soymilk.

Proteins contribute the greatest portion of total dry matter in soybeans, at approximately 40% w/w (Liu, 1997). However, sulphur-containing amino acids, including L-cysteine, are deficient in soybeans (Bressani & Elias, 1968; Liu, 1997). L-cysteine appears to be a growth limiting sulphur-containing amino acid for the propagation of bifidobacteria (Dave & Shah, 1997). L-cysteine is commonly incorporated into media used for the enumeration of bifidobacteria, as an amino nitrogen source and to reduce the redox potential of the medium, both of which favour the growth of anaerobic bifidobacteria (Shah, 2000). Kamaly (1997) found that the addition of L-cysteine to soymilk stimulated the growth of both *B. longum* and *B. bifidum*, with viable populations 5 times greater as compared to soymilk without L-cysteine after 24 h of incubation.

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Fermentation of plain soymilk by bifidobacteria hydrolysed isoflavone malonyl-, acetyl- and β-glucoside structures into bioactive aglycone forms, increasing the concentration of aglycones from less than 10% to approximately 50% of the total isoflavone content (Chapter 3.0). Supplementation of MRS broth with 1% (w/v) D-glucose enhanced the β-glucosidase activity of four out of a total of five strains of *Bifidobacterium* (Table 3.1); the enzyme required to hydrolyse isoflavone glucosides into aglycones. Hence, the extent of biotransformation (bacterial induced enzymic hydrolysis) from isoflavone glucosides to aglycone forms could be enhanced if the soymilk is supplemented with both D-glucose and L-cysteine.

The aims of this study were to examine the effects of D-glucose and L-cysteine supplementation on the growth of bifidobacteria in soymilk (made from SPI590) and the biotransformation of isoflavones by bifidobacteria.

4.2 MATERIALS AND METHODS

4.2.1 Bacteria

Pure cultures of BP20099, BB536, BL1941 and BB12 were obtained from the culture collections mentioned in section 3.2.1. These strains were selected for this study based on their ability to produce β-glucosidase (Table 3.1). Purity of cultures was checked using Gram staining and each strain was stored under the conditions described in section 3.2.1.

4.2.2 Bacterial growth media

Rehydrated MRS broth and MRS agar (de Mann *et al.* 1960) each containing additional 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine-HCl were prepared as described in section 3.2.2.

4.2.3 Manufacture of supplemented soymilk

Forty grams of SPI590 and 10 g of D-glucose per 1 L ultra-pure distilled water were used in the production of supplemented soymilk. Reconstitution of SPI590 and D-glucose followed by pH adjustment to 6.7 was the same as that described in section 3.2.3. Fourteen litres of supplemented soymilk was manufactured, dispensed into glass bottles and sterilised by autoclaving as described in section 3.2.3. Filter-sterilised L-cysteine solution (5% w/v) was also added to the supplemented soymilk (0.05% w/v final concentration) just prior to inoculation with bifidobacteria. The addition of L-cysteine, the use of glass bottles to minimize oxygen permeation, and the removal of dissolved oxygen from soymilk during heat treatment were measures used to establish a favourable growth environment for anaerobic bifidobacteria according to Collins & Hall (1984), Klaver *et al.* (1993) and Dave & Shah (1997). D-Glucose and L-cysteine were purchased from Sigma.

4.2.4 Fermentation of supplemented soymilk by bifidobacteria

Prior to soymilk fermentation studies, each strain of *Bifidobacterium* was activated as described in section 3.2.5. Five hundred millilitres of supplemented soymilk was then inoculated (in triplicate) with active culture of *Bifidobacterium* sp. (5% v/v) and incubated at 37°C for 48 h. Samples of 100 mL were withdrawn aseptically at 0, 12, 24, 36 and 48 h of incubation for enumeration of *Bifidobacterium* populations and the remainder stored at –20°C. Four hundred millilitres of non-inoculated sterile supplemented soymilk was also incubated for 48 h at 37°C (control) and 100 mL samples were withdrawn aseptically at 0, 24 and 48 h of incubation and stored at –20°C. Frozen samples of soymilk were freeze-dried as described in section 3.2.5 for the extraction of isoflavone and analysis using HPLC.

4.2.5 Enumeration of bifidobacteria in fermented supplemented soymilk

The pour plate method was used for the enumeration of viable populations of *Bifidobacterium*. The growth medium used for enumeration and incubation conditions are mentioned in section 3.2.6.

4.2.6 Extraction and HPLC analysis of isoflavones

The extraction of isoflavones, including malonyl-, acetyl-, β-glucosides, and aglycones, from non-fermented and fermented supplemented soymilk, was performed in duplicate using the method described in section 3.2.7. The preparation of mixed and single isoflavone standards used in HPLC analyses is described in section 3.2.8. Chromatographic analyses were carried out on a Hewlett Packard® 1100 series High Performance Liquid Chromatograph (Agilent Technologies) attached to a Keystone Scientific® ODS-C18 (250 x 4.6 mm internal diameter; 5 μm) reversed-phase column (section 3.2.9). All reagents used in the extraction of isoflavone and HPLC analyses were filtered through a 0.5 μm membrane (section 3.2.9). Gradient elution was used to isolate the isoflavones for detection as described in section 3.2.10, with the diode array UV/VIS detector set at a wavelength of 260 nm. The isoflavone isomers eluted in the order of malonyl-, β-, and acetyl-glucosides, followed by the aglycone forms of daidzein, glycitein and genistein at retention times shown in Figure 3.1. Quantification of isoflavone malonyl-, acetyl-, β-glucoside and aglycone isomers is also described in section 3.2.10. Isoflavone concentrations were calculated back to wet basis (expressed as mg per 100 mL of soymilk). Intra- and inter-assay %COV for the concentration of each isoflavone isomer in SPI590 was evaluated like that described in section 3.2.11, with the intra- and inter-assay %COV for each isoflavone isomer concentration in line with those found in previous work (section 3.2.11).

4.2.7 Statistical analysis

Growth of each *Bifidobacterium* strain for the fermentation of supplemented soymilk was performed in triplicate on two occasions. Enumeration of viable populations of bifidobacteria and quantification of isoflavones in fermented supplemented soymilk were performed in duplicate and are presented as a mean ± standard error of twelve replicates. A control of non-inoculated sterile supplemented soymilk was also incubated on both occasions. The isoflavone concentrations in the two control soymilks before and after incubation were also quantified in duplicate and the result presented as a mean ± standard error of four replicates. To find significant differences in the viable populations of *Bifidobacterium* sp.

and changes in isoflavone content in supplemented fermented soymilks, means were analysed with one-way ANOVA and 95% confidence intervals using Microsoft® Excel StatPro™ as described by Albright *et al.* (1999). In addition, viable populations of bifidobacteria and changes in isoflavone content between supplemented fermented soymilk and plain fermented soymilk (the latter reported in Chapter 3.0) were also analysed using ANOVA and 95% confidence intervals. ANOVA data with a $P<0.05$ was classified as statistically significant.

4.3 RESULTS AND DISCUSSION

4.3.1 Fermentation of supplemented soymilk by bifidobacteria

The supplementation of soymilk (made from soybeans) with D-glucose at a level of 1% (w/v) has shown conflicting results in influencing the growth of *Bifidobacterium* (Kamaly, 1997; Chou & Hou, 2000). Chou & Hou (2000) reported that the growth of *B. infantis* improved in soymilk supplemented with 1% (w/v) D-glucose after 24 and 48 h of incubation. In contrast, Kamaly (1997) reported that neither the growth of *B. longum* nor *B. bifidum* in soymilk was stimulated by the addition of 1% (w/v) D-glucose, with both strains showing greater increases in viable population in plain soymilk after 24 h of incubation. This may be due to variation in saccharolytic enzyme activity (Tochikura *et al.* 1986; Desjardins & Roy, 1990) and sugar utilisation patterns (Shah, 2000) observed between different strains of *Bifidobacterium*. Kamaly (1997) found that the addition of 0.05% (w/v) L-cysteine to soymilk (made from soybeans) increased the populations of bifidobacteria by 3.2 to 3.5 $\log_{10}$ CFU per mL after 24 h of incubation, compared to an increase of 2.5 to 2.6 $\log_{10}$ CFU per mL in plain soymilk. Consequently, both D-glucose and L-cysteine were added to soymilk made from SPI590 to determine whether they enhanced the growth of BP20099, BB536, BL1941 and BB12 in comparison to previous fermentation studies using plain soymilk (Chapter 3.0). Establishing a more favourable growth environment for BP20099, BB536, BL1941 and BB12 was an attempt to potentially enhance the enzymic transformation of isoflavone glucosides into bioactive aglycones, compared to that occurring in plain soymilk (Chapter 3.0).

Changes in the viable count of *Bifidobacterium* sp. grown in soymilk supplemented with 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine are shown in Table 4.1. BP20099 and BB536 showed the greatest increase in viable population when grown in supplemented soymilk and this occurred in the first 12 h of incubation. Similarly, BP20099 and BB536 also showed the greatest increase in viable population during 0 and 12 h of incubation when grown in plain soymilk (Table 3.2). Increases in the viable population of BP20099 and BB536 were significantly greater than those of BB12 and BL1941 grown in supplemented soymilk during the first 12 h of incubation ($P<0.05$) (Table 4.1); similar to that found in plain soymilk (Table 3.2). BB12 grown in supplemented soymilk increased by 0.32 $\log_{10}$ CFU per mL in the first 12 h of incubation (Table 4.1), lower than the increase of 0.78 $\log_{10}$ CFU per mL observed in plain soymilk (Table 3.2). BL1941 consistently showed the poorest growth in both supplemented soymilk (Table 4.1) and plain soymilk (Table 3.2) during the first 12 h of incubation. When comparing the growth of BP20099, BB536 and BB12 in supplemented soymilk (Table 4.1) to plain soymilk (Table 3.2) over the first 12 h of incubation, the addition of D-glucose and L-cysteine did not significantly enhance bacterial growth ($P>0.05$).

The addition of D-glucose and L-cysteine did significantly enhance the growth of BB12 over 0 and 24 h of incubation ($P<0.05$) (Table 4.1). There appeared to be a longer lag phase for BB12 when grown in supplemented soymilk (Table 4.1) compared to plain soymilk (Table 3.2). The exponential growth phase of BB12 in supplemented soymilk occurred during 12 and 24 h of incubation, increasing by 1.27 $\log_{10}$ CFU per mL (Table 4.1). The increase of 1.59 $\log_{10}$ CFU per mL in the viable population of BB12 in supplemented soymilk between 0 and 24 h of incubation (Table 4.1) was significantly higher than the increase of 0.93 $\log_{10}$ CFU per mL occurring in plain soymilk (Table 3.2) ($P<0.05$). In addition, the increase in the viable population of BB12 in supplemented soymilk over 12 and 24 h of incubation was at the same level as the increase shown by BP20099 and BB536 during their exponential growth phase between 0 and 12 h of incubation (Table 4.1).

In supplemented soymilk, the viable population of BL1941 decreased from 6.37 to 5.80 $\log_{10}$ CFU per mL over 0 and 12 h of incubation, which was followed by an increase of 0.78 $\log_{10}$ CFU per mL between 12 and 24 h of incubation (Table 4.1). Therefore, between 0 and 24 h of incubation, BL1941 only showed an increase of 0.21 $\log_{10}$ CFU per mL. This increase is not enough evidence to suggest that this strain could grow in soymilk supplemented with D-glucose and L-cysteine. BL1941 also was unable to grow in plain soymilk, with a reduction in viable count after 24 h of incubation (Table 3.2).

During 24 and 48 h of incubation, BP20099, BB536 and BB12 exhibited better survival rates in supplemented soymilk (Table 4.1) compared to plain soymilk (Table 3.2). BP20099, BB536 and BB12 depicted increases in viable population of 0.57, 0.21 and 0.29 $\log_{10}$ CFU per mL, respectively, between 24 and 48 h of incubation in supplemented soymilk (Table 4.1). In plain soymilk, each of these strains decreased in viable population between 24 and 48 h of incubation (Table 3.2). The extended growth phase observed in the supplemented soymilk may have been due to the additional D-glucose supply. In contrast, the death phase for BP20099, BB536 and BB12 incubated in plain soymilk occurred after 24 h of incubation (Table 3.2), which was most likely due to a diminished nutrient supply.

4.3.2 HPLC analysis of isoflavones in supplemented non-fermented soymilk

Reports suggest that there is a considerable variation in isoflavone content and isomer composition in soybeans, soy ingredients and soy foods due to differences in agricultural practices, used in soybean cultivation (Wang & Murphy, 1994; Tsukamoto *et al.* 1995), and processing steps used in manufacture (Wang & Murphy, 1996). The plain non-fermented soymilk reported in Chapter 3.0 contained a total isoflavone content of 4.956 mg per 100 mL (Tables 3.3 to 3.6), while the supplemented soymilk made from a different batch of SPI590 contained a slightly higher total isoflavone content of 5.245 mg per 100 mL. However, there was no significant difference in total isoflavone content between the supplemented soymilk used in this study and plain soymilk reported earlier (Chapter 3.0).

The total concentration of isoflavone aglycone forms found in the supplemented non-fermented soymilk (prior to inoculation with pure culture of *Bifidobacterium* sp.) was 0.520 mg per 100 mL. In plain non-fermented soymilk (non-inoculated), the isoflavone aglycone concentration was 0.381 mg per 100 mL (Tables 3.3 to 3.6). The bioactive aglycone structures only contributed 8 to 10% of the total isoflavone content in both supplemented and plain non-fermented soymilk. The β-glucoside forms contributed the greatest concentration of isoflavone isomers in the supplemented non-fermented soymilk at 4.251 mg per 100 mL (81% of total). Similarly, the concentration of isoflavone β-glucosides in plain non-fermented soymilk was 4.111 mg per 100 mL (83% of total) (Tables 3.3 to 3.6). The concentration of isoflavone aglycone was significantly lower than that of isoflavone β-glucoside in both the plain and supplemented non-fermented soymilk ($P<0.05$). Murphy *et al.* (1999) and King & Bignell (2000) also found that the isoflavone β-glucosides were the predominant isomeric forms in non-fermented soymilk. Isoflavone glucosides derived from soymilk require bacterial-induced enzymic hydrolysis to convert them into a bioavailable aglycone form. This process increases their retention in the gastrointestinal tract (Setchell *et al.* 2001). Izumi *et al.* (2000) discovered that isoflavone aglycones were absorbed faster and in higher amounts than their respective β-glucoside forms. Nevertheless, non-fermented soymilk may still be a better source of isoflavone compared to solid, non-fermented soy foods, as it has been observed that isoflavone absorption and subsequent urinary excretion of isoflavone metabolites is more rapid when isoflavones are derived from a liquid food (Setchell, 2000).

The concentration of isoflavone malonylglucosides found in supplemented non-fermented soymilk (non-inoculated) was 0.098 mg per 100 mL. This was equivalent to the concentration of malonylglucosides found in plain soymilk ($P>0.05$), at 0.078 mg per 100 mL (Tables 3.3 to 3.6). The concentration of malonylglucosides comprised less than 2% of the total isoflavone content found in both soymilks. King & Bignell (2000) also found very low levels of malonylgenistin (approximately 1 to 2% of total isoflavone concentration) in a variety of soymilks. In contrast, Murphy *et al.* (1999) detected relatively high levels of malonylglucosides in soymilk, ranging from 6 to 22% of the total

isoflavone content. The variation in isoflavone malonylglucoside concentration observed between this study and that reported by Murphy *et al.* (1999) is due possibly to the type of soybean or soy ingredient used in soymilk manufacture and/or the type of processing used in soymilk preparation. SPI was used to prepare the soymilk in this study, which is known to have a modified isoflavone content and composition due to losses caused by defatting and insoluble carbohydrate extraction (Wang & Murphy, 1996). Murphy *et al.* (1999) reported that ultra high temperature (UHT) treated (145°C for 1 to 3 sec) soymilk had a significantly lower concentration of malonylglucosides compared with pasteurised soymilk ($P<0.05$). The chemical transformation of isoflavone malonylglucoside conjugates was previously reported by Barnes *et al.* (1994), suggesting that malonylglucosides are prone to both heat-induced decarboxylation to form acetylglucosides, and de-esterification to form β-glucosides. However, in a parallel experiment we quantified the isoflavone isomer composition of soymilk made from 4% (w/v) SPI590 before and after sterilisation at 121°C for 15 min and found that there was no change in isoflavone composition under these conditions. Hence, the low proportion of isoflavone malonylglucoside in the plain (Tables 3.3 to 3.6) and supplemented non-fermented soymilk was due to the low concentration of malonylglucoside found in the SPI used in manufacture.

Like that in plain non-fermented soymilk (Chapter 3.0), the concentration of isoflavone acetylglucoside in supplemented non-fermented soymilk, prior to bacterial inoculation, was equivalent to the aglycone level, at approximately 8% of total isoflavone. An acetylglucoside level of 0.376 mg per 100 mL was found in supplemented non-fermented soymilk. The levels reported in this study are higher than those reported by Murphy *et al.* (1999) and King & Bignell (2000). King & Bignell (2000) did not detect any acetylglucoside isomers in soymilk samples, whereas Murphy *et al.* (1999) only detected acetylgenistin at approximately 4% of total isoflavone.

Of the aglycone isomers in supplemented non-fermented soymilk, the concentration of genistein, at 0.280 mg per 100 mL, was significantly higher than both daidzein (0.161 mg per 100 mL) and glycitein

(0.079 mg per 100 mL) ($P<0.05$). In plain non-fermented soymilk, 0.202 mg of genistein per 100 mL was also significantly higher than the 0.120 mg of daidzein and 0.059 mg of glycitein per 100 mL (Tables 3.3 to 3.6) ($P<0.05$). This is consistent with the data of Murphy *et al.* (1999), showing that the concentration of genistein was higher than the aglycone forms of daidzein and glycitein in soymilk. Amongst the β-glucoside isomers, the concentration of genistin was significantly higher than daidzin and glycitin in supplemented and plain non-fermented soymilk ($P<0.05$). The concentration of genistin, daidzin and glycitin in supplemented non-fermented soymilk was 2.783, 1.078 and 0.390 mg per 100 mL, respectively; equivalent to the levels of these isomers in plain non-fermented soymilk (Tables 3.3 to 3.6). King & Bignell (2000) also found that the concentration of genistin was consistently higher than all other isoflavone isomers.

A control sample of non-inoculated sterile supplemented soymilk was incubated for 48 h and analysed for its isoflavone aglycone, malonyl-, acetyl- and β-glucoside concentrations to determine whether any chemical transformation in isoflavone isomers was occurring in a non-fermented soymilk during incubation. The total isoflavone concentration did not significantly change in the control soymilk to suggest there was any deconjugation of isoflavone glucosides occurring, with a level of 5.159 mg per 100 mL after 24 h of incubation and 5.198 mg per 100 mL after 48 h ($P>0.05$). Both reductions in isoflavone concentration after 24 and 48 h of incubation were less than a 2% deviation from the sample extracted at 0 h (5.245 mg per 100 mL), and are most likely analytical errors encountered in HPLC analysis. Furthermore, the concentration of isoflavone aglycone and β-glucoside in the control soymilk did not significantly change after 24 and 48 h of incubation ($P>0.05$). At 0 h, the concentration of aglycone was 0.520±0.010 mg per 100 mL, after 24 h it was 0.512±0.013 mg per 100 mL, and then 0.523±0.007 mg per 100 mL after 48 h of incubation. The concentration of isoflavone β-glucoside decreased slightly from 4.251±0.068 mg per 100 mL (at 0 h) to 4.213±0.053 and 4.216±0.050 mg per 100 mL after 24 and 48 h of incubation, respectively. The level of isoflavone malonylglucoside and acetylglucoside in the control soymilk also fluctuated slightly after 24 and 48 h of incubation, but not

enough to suggest there was any chemical transformation occurring. Malonylglucoside concentrations in the control soymilk at 0, 24 and 48 h of incubation were 0.098±0.003, 0.089±0.005 and 0.093±0.005 mg per 100 mL, respectively. The level of acetylglucoside was 0.376±0.005, 0.365±0.009 and 0.366±0.007 mg per 100 mL soymilk (control) at 0, 24 and 48 h of incubation, respectively.

4.3.3 Biotransformation of isoflavones by bifidobacteria in supplemented fermented soymilk

The change in concentration of isoflavone aglycone, β-glucoside, malonylglucoside and acetylglucoside in supplemented soymilk after 48 h of incubation with *Bifidobacterium* sp. are shown in Table 4.2. The fermentation of supplemented soymilk by BP20099, BB536 and BB12 significantly increased the concentration of isoflavone aglycone after 24 h of incubation ($P<0.05$), by possibly deconjugating the isoflavone glucosides via β-glucosidase induced hydrolysis. In Chapter 3.0, it was reported that the significant increase in aglycone concentration in plain soymilk fermented by *Bifidobacterium* sp. ($P<0.05$) was due predominantly to the hydrolysis of isoflavone β-glucosides and to a lesser extent malonyl- and acetyl-glucosides. For the first 12 h of incubation, supplemented soymilk fermented by BB12 showed the greatest increase in aglycone concentration, from 0.578 to 1.560 mg per 100 mL (Table 4.2), but was significantly lower than the increase in aglycone level occurring in plain soymilk fermented by the same strain (Table 3.6) ($P<0.05$). Additionally, plain soymilk fermented by BP20099 and BB536 (Tables 3.3 and 3.4, respectively), also showed a significantly greater increase in aglycone compared to supplemented soymilk (Table 4.2) after 12 h of incubation ($P<0.05$). Supplemented soymilk fermented by BP20099 and BB536 for 12 h only increased the aglycone concentration from 0.511 to 0.662 and from 0.523 to 0.622 mg per 100 mL, respectively (Table 4.2). In plain soymilk, incubation of BP20099 and BB536 for 12 h increased the concentration of aglycone by 0.778 (Table 4.3) and 0.696 (Table 4.4) mg per 100 mL, respectively. Based on these observations, the addition of D-glucose did not seem to favour the enzymic hydrolysis of isoflavone glucosides into aglycone in soymilk fermented by *Bifidobacterium*. As shown in Table 3.1, BP20099, BB536 and BB12 produced significantly greater levels of β-glucosidase when additional 1% (w/v) D-glucose was added to MRS

broth ($P<0.05$). However, MRS broth contains 2% (w/v) glucose whereas soymilk contains predominantly α-galactosyl oligosaccharides, which may stimulate the production of α-galactosidase needed to metabolise these sugars.

Between 12 and 24 h of incubation, fermentation of supplemented soymilk by BP20099 and BB536 increased the concentration of aglycone from 0.662 to 0.982 and 0.622 to 0.936 mg per 100 mL, respectively (Table 4.2). Once again, the increase in aglycone concentration occurring in plain soymilk fermented by BP20099 and BB536 (Tables 3.3 and 3.4, respectively) was significantly greater than that in supplemented soymilk fermented by the same strains ($P<0.05$). In contrast, during 12 and 24 h of incubation, fermentation of supplemented soymilk by BB12 resulted in a significantly greater increase in aglycone concentration compared to that in plain soymilk ($P<0.05$). This may be associated to BB12's longer lag phase when grown in supplemented soymilk, with logarithmic growth and greater increases in viable population occurring between 12 and 24 h of incubation (Table 4.1). Between 24 and 48 h of incubation, there was no significant difference in the increase in aglycone concentration between supplemented soymilk (Table 4.2) and plain soymilk fermented by BP20099 (Table 3.3), BB536 (Table 3.4) and BB12 (Table 3.6).

With the increase in aglycone concentration, fermentation of supplemented soymilk by BP20099, BB536 and BB12 significantly decreased the concentration of isoflavone β-glucoside after 24 h of incubation ($P<0.05$) (Table 4.2). However, the decrease in concentration of isoflavone β-glucoside was significantly greater in plain soymilk fermented by BP20099 (Table 3.3), BB536 (Table 3.4) and BB12 (Table 3.6) ($P<0.05$). In supplemented soymilk, the greatest decrease in the concentration of isoflavone β-glucoside was from 4.098 to 2.630 mg per 100 mL after 24 h of fermentation by BB12 (Table 4.2). In contrast, plain soymilk fermented by BB12 reduced the concentration of isoflavone β-glucoside from 4.111 (at 0 h) to 0.661 (at 24 h) mg per 100 mL (Table 3.6). This suggests that due to a lack of available simple sugars, as in the case of the plain soymilk, bifidobacteria scavenge for alternative carbon sources

such as the glucose moiety of isoflavone glucoside. Whereas in supplemented soymilk containing additional 1% (w/v) D-glucose, bifidobacteria can readily utilise the free glucose as a growth substrate. Furthermore, addition of L-cysteine in order to reduce the redox potential of the soymilk, establishing a more favourable growth environment for anaerobic bifidobacteria, did not enhance the biotransformation of isoflavone glucosides into aglycone. Nevertheless, during 24 and 36 h of incubation, the decrease in isoflavone β-glucoside in supplemented soymilk fermented by BP20099 and BB12 (Table 4.2) was significantly greater than the reduction in β-glucoside occurring in plain soymilk fermented by the same strains (Tables 3.3 and 3.6) ($P<0.05$). It appeared that supplementation of soymilk with D-glucose and L-cysteine established a more favourable environment for bifidobacteria during 24 and 48 h of incubation, improving their survival compared to that in plain soymilk, which may have enhanced the hydrolysis of isoflavone β-glucoside by BP20099 and BB12 between 24 and 36 h.

The enzymic hydrolysis of malonyl- and acetyl-glucosides due to fermentation by BP20099, BB536 and BB12 significantly decreased the concentration of these isomers in supplemented soymilk after 24 h of incubation ($P<0.05$) (Table 4.2). However, there was no significant difference between the reduction in concentration of malonyl- and acetyl-glucoside occurring in supplemented soymilk (Table 4.2) compared to plain soymilk (Tables 3.3, 3.4 and 3.6) ($P>0.05$). Hence, D-glucose and L-cysteine supplementation did not appear to enhance the biotransformation of malonyl- and acetyl-glucosides into aglycone. Additionally, the malonyl- and acetyl-glucosides that may have hydrolysed during fermentation contributed very little to the increase in aglycone concentration, due to the relatively low levels of these isomers found in the original non-fermented soymilk. The greatest reduction in malonylglucoside and possible release of aglycone occurred in supplemented soymilk fermented by BP20099, decreasing from 0.108 to 0.054 mg per 100 mL after 24 h of incubation (Table 4.2). The greatest reduction in acetylglucoside and possible release of aglycone was only by 0.144 mg per 100 mL in supplemented soymilk fermented by BB12 for 12 h (Table 4.2).

In supplemented soymilk fermented by BP20099, BB536 and BB12, the concentration of the aglycones daidzein, genistein and glycitein significantly increased after 24 h of incubation ($P<0.05$) (Table 4.3). In parallel, there was a significant decrease in the concentration of the β-glucosides daidzin, genistin and glycitin in supplemented soymilk ($P<0.05$) (Table 4.4). However, the reduction in concentration of daidzin, genistin and glycitin due to enzymic hydrolysis was significantly greater in plain soymilk ($P<0.05$) for BP20099 (Table 3.3), BB536 (Table 3.4) and BB12 (Table 3.6). Hence, the greater hydrolysis of each of these β-glucoside isomers in plain soymilk also resulted in a significantly greater increase in the concentration of daidzein, genistein and glycitein compared to supplemented soymilk ($P<0.05$). The hydrolytic transformation of genistin to form genistein contributed the greatest portion to the total aglycone concentration in supplemented soymilk after fermentation. The hydrolysis of glycitin, which is commonly found at lower concentrations in comparison to daidzin and genistin in soy foods (Murphy *et al.* 1999; King & Bignell, 2000), only increased the concentration of glycitein in the range of 0.018 to 0.091 mg per 100 mL of supplemented soymilk after 24 of incubation (Table 4.3). Glycitein contributed the smallest portion to the total aglycone concentration after fermentation by bifidobacteria in both plain and supplemented soymilk.

In contrast to the potential isoflavone glucoside hydrolysing activities of BP20099, BB536 and BB12, the fermentation of supplemented soymilk by BL1941 did not significantly reduce the concentration of isoflavone malonyl-, acetyl- and β-glucoside ($P>0.05$) (Table 4.2). Hence, there was no significant increase in the concentration of aglycone in supplemented soymilk fermented by BL1941, even after the entire 48 h of incubation ($P>0.05$) (Tables 4.2 and 4.3).

Results of viable populations of *Bifidobacterium* (Table 4.1) and the concentration of isoflavone isomers before and after fermentation (Tables 4.2 to 4.4) revealed correlations between bacterial growth and enzymic hydrolysis of isoflavones. Generally, biotransformation of isoflavone glucosides into aglycone was significantly higher during the first 24 h of incubation (or the exponential growth phase)

($P<0.05$). Nevertheless, a greater increase in the viable population of bifidobacteria in supplemented soymilk did not correlate with more extensive biotransformation of isoflavone glucosides into aglycone, similar to that occurring in plain soymilk (Chapter 3.0). BB12, when grown in supplemented soymilk, showed a significantly greater increase in aglycone concentration compared to BP20099 and BB536 during 0 and 24 h of incubation ($P<0.05$) (Table 4.2), coinciding with its highest increase in viable count (Table 4.1). However, BB12's increase in viable population after 24 h of incubation was lower than the increase of BP20099 and BB536 in supplemented soymilk. BB12 also showed a significantly higher level of β-glucosidase production compared to BP20099 and BB536 ($P<0.05$) (Table 3.1), which may have reflected the significantly greater decrease in isoflavone β-glucoside in supplemented soymilk fermented by BB12 ($P<0.05$) (Table 4.2).

4.4 CONCLUSIONS

The effect of D-glucose and L-cysteine supplementation on the viability of *Bifidobacterium* sp. in soymilk varied considerably between strains and incubation period. In comparison to plain soymilk, D-glucose and L-cysteine supplementation did not enhance the growth of bifidobacteria in the first 12 h of incubation. However, growth was enhanced from 12 to 24 h of incubation and their survival improved between 24 and 48 h. Addition of D-glucose and L-cysteine to soymilk did not enhance the biotransformation of isoflavone glucosides into bioactive aglycone. The concentration of isoflavone aglycone in plain soymilk fermented by BP20099, BB536 and BB12 was higher after 24 h of incubation compared to that in supplemented soymilk. This coincided with a greater reduction in the concentration of isoflavone β-glucoside in plain soymilk. On the whole, these results indicate that soymilk does not require supplementation with D-glucose and L-cysteine to enhance the biotransformation of isoflavone glucosides into bioactive forms.

Table 4.1 Changes in viable count (log_{10} CFU per mL) of *Bifidobacterium* sp. incubated in supplemented[1] soymilk for 12, 24, 36 and 48 h at 37°C (mean ± standard error; n = 12)

Bifidobacteria Strains	**Change in log_{10} CFU per mL between intervals[2] (n = 12)**								
	0 h		**12 h**		**24 h**		**36 h**		**48 h**
BP20099	6.54±0.04	$+1.50^{a}$	8.04±0.03	$+0.44^{c}$	8.48±0.07	$+0.27^{a}$	8.75±0.06	$+0.30^{a}$	9.05±0.08
BB536	6.66±0.05	$+1.31^{a}$	7.97±0.02	$+0.50^{bc}$	8.47±0.08	$+0.20^{a}$	8.67±0.10	$+0.01^{ab}$	8.68±0.09
BL1941	6.37±0.06	-0.57^{c}	5.80±0.02	$+0.78^{abc}$	6.58±0.24	$+0.54^{a}$	7.12±0.01	-0.03^{b}	7.09±0.13
BB12	7.09±0.03	$+0.32^{b}$	7.41±0.32	$+1.27^{a}$	8.68±0.09	$+0.18^{a}$	8.86±0.10	$+0.11^{ab}$	8.97±0.13

BP20099: *B. pseudolongum*; BB536: *B. longum*; BL1941: *B. longum*; BB12: *B. animalis*.

Means in the same column with different superscript are significantly different ($P<0.05$).

[1]Soymilk supplemented with 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine.

[2]Mean of the difference in log_{10} CFU per mL between incubation time intervals.

Table 4.2 Concentration of isoflavone aglycone, β-glucoside, malonylglucoside and acetylglucoside (mg per 100 mL) in supplemented[1] soymilk fermented by *Bifidobacterium* sp. for 12, 24, 36 and 48 h at 37°C (mean ± standard error; n = 12)

Isoflavone Isomers	Bifidobacteria Strains	Change in isoflavone isomer concentration between intervals[2] (n = 12)								
		0 h[3]		12 h		24 h		36 h		48 h
Aglycone[4]	BP20099	0.511±0.017	+0.151^{bcd}	0.662±0.011	+0.320^{bc}	0.982±0.053	+0.597^{a}	1.579±0.026	+0.189^{a}	1.768±0.046
	BB536	0.523±0.015	+0.099^{cd}	0.622±0.016	+0.314^{c}	0.936±0.060	+0.669^{a}	1.605±0.039	+0.140^{a}	1.745±0.064
	BL1941	0.535±0.021	−0.040^{d}	0.495±0.006	−0.035^{d}	0.460±0.007	+0.032^{b}	0.492±0.006	+0.015^{a}	0.507±0.008
	BB12	0.578±0.062	+0.982^{a}	1.560±0.108	+0.796^{a}	2.356±0.146	+0.545^{a}	2.901±0.051	+0.183^{a}	3.084±0.041
β-Glucoside[5]	BP20099	4.198±0.077	−0.250^{a}	3.948±0.079	−0.192^{a}	3.756±0.073	−1.308^{d}	2.447±0.080	−0.342^{bc}	2.105±0.058
	BB536	4.273±0.067	−0.259^{a}	4.014±0.054	−0.259^{a}	3.755±0.059	−0.934^{bcd}	2.821±0.085	−0.344^{c}	2.477±0.079
	BL1941	4.213±0.091	+0.049^{a}	4.262±0.053	−0.198^{a}	4.064±0.052	+0.322^{a}	4.386±0.032	+0.198^{a}	4.584±0.042
	BB12	4.098±0.108	−1.468^{b}	2.630±0.107	−0.956^{b}	1.674±0.251	−1.091^{cd}	0.583±0.085	−0.121^{abc}	0.462±0.010
Malonylglucoside[6]	BP20099	0.108±0.026	−0.038^{c}	0.070±0.007	−0.016^{a}	0.054±0.008	+0.002^{a}	0.056±0.005	−0.017^{b}	0.039±0.001
	BB536	0.087±0.017	−0.011^{abc}	0.076±0.007	−0.019^{a}	0.057±0.007	−0.016^{a}	0.041±0.002	−0.003^{ab}	0.038±0.001
	BL1941	0.103±0.035	−0.008^{a}	0.095±0.002	−0.001^{a}	0.094±0.002	+0.001^{a}	0.095±0.002	+0.007^{a}	0.101±0.002
	BB12	0.082±0.013	−0.021^{bc}	0.061±0.003	−0.008^{a}	0.053±0.004	−0.015^{a}	0.038±0.001	+0.005^{a}	0.043±0.004
Acetylglucoside[7]	BP20099	0.362±0.012	−0.028^{a}	0.334±0.018	−0.008^{a}	0.326±0.022	−0.190^{c}	0.136±0.012	+0.003^{a}	0.139±0.004
	BB536	0.345±0.015	−0.049^{a}	0.296±0.015	−0.026^{a}	0.270±0.014	−0.153^{abc}	0.117±0.011	−0.004^{a}	0.113±0.005
	BL1941	0.350±0.022	−0.039^{a}	0.311±0.009	+0.009^{a}	0.320±0.014	−0.066^{a}	0.254±0.017	+0.033^{a}	0.287±0.007
	BB12	0.368±0.011	−0.144^{b}	0.224±0.012	−0.017^{a}	0.207±0.017	−0.170^{bc}	0.037±0.003	+0.004^{a}	0.041±0.006

BP20099: *B. pseudolongum*; BB536: *B. longum*; BL1941: *B. longum*; BB12: *B. animalis*.

One-way ANOVA of means arranged as a column of 4 values. Means in the same column with different superscript are significantly different ($P<0.05$).

[1]Soymilk supplemented with 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine.

[2]Mean of the difference in isoflavone aglycone, β-glucoside, malonylglucoside and acetylglucoside concentrations between incubation times.

[3]Concentration of isoflavone isomers in non-fermented soymilk (inoculated with pure culture of *Bifidobacterium* sp.).

[4]Mean total of aglycone isomers (daidzein, genistein and glycitein).

[5]Mean total of β-glucoside isomers (daidzin, genistin and glycitin).

[6]Mean total of malonylglucoside isomers (malonyldaidzin, malonylgenistin and malonylglycitin).

[7]Mean total of acetylglucoside isomers (acetyldaidzin, acetylgenistin and acetylglycitin).

Table 4.3 Concentration of isoflavone aglycone isomers (mg per 100 mL) in supplemented[1] soymilk fermented by *Bifidobacterium* sp. for 12, 24, 36 and 48 h at 37°C (mean ± standard error; n = 12)

Isoflavone Isomers	Bifidobacteria Strains	Change in isoflavone isomer concentrations between intervals[2] (n = 12)								
		0 h[3]		12 h		24 h		36 h		48 h
Daidzein	BP20099	0.164±0.008	+0.011^{bcd}	0.175±0.003	+0.060^{bcd}	0.235±0.010	+0.078^{a}	0.313±0.004	+0.031^{a}	0.344±0.008
	BB536	0.157±0.007	+0.008^{cd}	0.165±0.002	+0.058^{cd}	0.223±0.010	+0.097^{a}	0.320±0.007	+0.022^{a}	0.342±0.012
	BL1941	0.160±0.004	−0.010^{d}	0.150±0.003	−0.010^{d}	0.140±0.003	+0.008^{b}	0.148±0.003	0.000^{a}	0.148±0.003
	BB12	0.184±0.011	+0.155^{a}	0.339±0.017	+0.137^{a}	0.476±0.016	+0.121^{a}	0.597±0.011	+0.024^{a}	0.621±0.008
Genistein	BP20099	0.272±0.009	+0.135^{bcd}	0.407±0.008	+0.237^{bc}	0.644±0.040	+0.503^{a}	1.147±0.022	+0.147^{a}	1.294±0.036
	BB536	0.292±0.011	+0.086^{cd}	0.378±0.013	+0.234^{c}	0.612±0.046	+0.547^{a}	1.159±0.030	+0.112^{a}	1.271±0.048
	BL1941	0.292±0.009	−0.020^{d}	0.272±0.003	−0.022^{d}	0.250±0.004	+0.021^{b}	0.271±0.003	+0.013^{b}	0.284±0.004
	BB12	0.323±0.015	+0.776^{a}	1.099±0.086	+0.619^{a}	1.718±0.126	+0.416^{a}	2.134±0.036	+0.094^{a}	2.228±0.031
Glycitein	BP20099	0.075±0.003	+0.005^{bcd}	0.080±0.001	+0.023^{ab}	0.103±0.005	+0.016^{bc}	0.119±0.001	+0.010^{a}	0.129±0.003
	BB536	0.074±0.002	+0.004^{cd}	0.078±0.001	+0.014^{bc}	0.092±0.003	+0.034^{abc}	0.126±0.002	+0.006^{a}	0.132±0.005
	BL1941	0.083±0.005	−0.009^{d}	0.074±0.001	−0.004^{c}	0.070±0.002	+0.003^{c}	0.073±0.001	+0.002^{a}	0.075±0.001
	BB12	0.071±0.002	+0.051^{a}	0.122±0.005	+0.040^{a}	0.162±0.005	+0.061^{a}	0.223±0.007	+0.012^{a}	0.235±0.005

BP20099: *B. pseudolongum*; BB536: *B. longum*; BL1941: *B. longum*; BB12: *B. animalis*.

One-way ANOVA of means arranged as a column of 4 values. Means in the same column with different superscript are significantly different ($P<0.05$).

[1]Soymilk supplemented with 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine.

[2]Mean of the difference in isoflavone aglycone isomer concentrations between incubation times.

[3]Concentration of isoflavone aglycone isomers in non-fermented soymilk (inoculated with pure culture of *Bifidobacterium* sp.).

Table 4.4 Concentration of isoflavone β-glucoside isomers (mg per 100 mL) in supplemented[1] soymilk fermented by *Bifidobacterium* sp. for 12, 24, 36 and 48 h at 37°C (mean ± standard error; n = 12)

Isoflavone Isomers	Bifidobacteria Strains	Change in isoflavone isomer concentrations between intervals[2] (n = 12)								
		0 h[3]		12 h		24 h		36 h		48 h
Daidzin	BP20099	1.068±0.013	-0.078^{a}	0.990±0.020	-0.019^{a}	0.971±0.024	-0.309^{cd}	0.662±0.020	-0.083^{c}	0.579±0.012
	BB536	1.086±0.009	-0.078^{a}	1.008±0.014	-0.056^{a}	0.952±0.016	-0.204^{bc}	0.748±0.022	-0.080^{bc}	0.668±0.023
	BL1941	1.070±0.013	$+0.013^{a}$	1.083±0.014	-0.051^{a}	1.032±0.013	$+0.069^{a}$	1.101±0.010	$+0.052^{a}$	1.153±0.011
	BB12	1.066±0.009	-0.391^{b}	0.675±0.026	-0.204^{b}	0.471±0.049	-0.346^{d}	0.125±0.005	-0.017^{abc}	0.108±0.004
Genistin	BP20099	2.733±0.041	-0.154^{a}	2.579±0.052	-0.215^{a}	2.364±0.048	-0.902^{d}	1.462±0.057	-0.245^{c}	1.217±0.045
	BB536	2.786±0.032	-0.164^{a}	2.622±0.036	-0.208^{a}	2.414±0.046	-0.694^{cd}	1.720±0.057	-0.243^{bc}	1.477±0.051
	BL1941	2.763±0.030	$+0.021^{a}$	2.784±0.035	-0.129^{a}	2.655±0.036	$+0.234^{a}$	2.889±0.022	$+0.123^{a}$	3.012±0.030
	BB12	2.650±0.051	-1.048^{b}	1.602±0.080	-0.736^{b}	0.866±0.194	-0.654^{bcd}	0.212±0.087	-0.101^{abc}	0.111±0.004
Glycitin	BP20099	0.397±0.011	-0.019^{a}	0.378±0.008	$+0.043^{a}$	0.421±0.017	-0.097^{c}	0.324±0.005	-0.015^{a}	0.309±0.003
	BB536	0.401±0.010	-0.016^{a}	0.385±0.007	$+0.004^{abc}$	0.389±0.008	-0.037^{abc}	0.352±0.008	-0.020^{a}	0.332±0.008
	BL1941	0.380±0.016	$+0.015^{a}$	0.395±0.007	-0.018^{c}	0.377±0.006	$+0.019^{a}$	0.396±0.004	$+0.023^{a}$	0.419±0.005
	BB12	0.382±0.008	-0.030^{a}	0.352±0.003	-0.016^{bc}	0.336±0.011	-0.090^{bc}	0.246±0.005	-0.002^{a}	0.244±0.010

BP20099: *B. pseudolongum*; BB536: *B. longum*; BL1941: *B. longum*; BB12: *B. animalis*.

One-way ANOVA of means arranged as a column of 4 values. Means in the same column with different superscript are significantly different ($P<0.05$).

[1]Soymilk supplemented with 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine.

[2]Mean of the difference in isoflavone β-glucoside isomer concentrations between incubation times.

[3]Concentration of isoflavone β-glucoside isomers in non-fermented soymilk (inoculated with pure culture of *Bifidobacterium* sp.).

5.0 Metabolism of Oligosaccharides and Aldehydes and Production of Organic Acids in Soymilk by Probiotic Bifidobacteria

5.1 INTRODUCTION

The 'beany' flavour of soymilk is predominantly due to the presence of hexanal and pentanal. The formation of these aldehydes mainly results from the hydroperoxidation of polyunsaturated fatty acids, catalysed by lipoxygenases (Wilkens *et al.* 1967; Wilkens & Lin, 1970). Soymilk also contains oligosaccharides, raffinose and stachyose, which are indigestible due to the absence of α-galactosidase in the small intestinal mucosa of humans. Consequently, intact oligosaccharides pass directly into the distal intestinal area (ileum and colon) where they are metabolised by bacteria that possess the enzyme, resulting in the production of gases (Cristofaro *et al.* 1974). Hence, soybean oligosaccharides are commonly associated with stomach discomfort and flatulence (Rackis *et al.* 1970). Nevertheless, oligosaccharides have been shown to possess prebiotic properties, by increasing the populations of indigenous bifidobacteria in the colon (Hayakawa *et al.* 1990).

Bifidobacteria produce a number of saccharolytic enzymes (Desjardins & Roy, 1990). Scalabrini *et al.* (1998) discovered that all twenty-seven strains of bifidobacteria analysed produced varying levels of α-galactosidase after 12, 24 and 48 h of incubation. Thus, these strains were able to metabolise α-galactosyl oligosaccharides when grown in soymilk made from soybeans (Scalabrini *et al.* 1998). In addition, fermentation of commercial soymilks by bifidobacteria have been shown to reduce the levels of hexanal and pentanal, to varying degrees, after 24 h of incubation (Scalabrini *et al.* 1998; Desai *et al.* 2002).

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During growth, bifidobacteria exclusively degrade hexoses (Scardovi & Trovatelli, 1965), with the fermentation of 2 moles of glucose generally leading to the formation of 3 moles of acetate and 2 moles of L (+)-lactate in synthetic medium (Biavati *et al.* 1992). Studies conducted on soymilk (made from soybeans) by Scalabrini *et al.* (1998) and Hou *et al.* (2000) showed considerable variation in lactic and acetic acid production between different strains of bifidobacteria, with higher levels of acetic acid found in fermented soymilk.

According to Shurtleff & Aoyagi (1984), soymilk made from SPI has a reduced level of beaniness, attributed to the removal of its lipid fraction, and a lower level of indigestible oligosaccharide due to losses during protein isolation. Nevertheless, SPIs are still associated with an unfavourable odour and flavour, caused predominantly by the presence of hexanal (Inouye *et al.* 2002). To date, there have been no fermentation studies undertaken to investigate the metabolic effects of commercial *Bifidobacterium* strains on the oligosaccharide and aldehyde content of soymilk made from SPI. As reported in Chapter 3.0, it was found that β-glucosidase-producing *Bifidobacterium* sp. hydrolysed glucosidic structures of isoflavone in soymilk made from SPI590. Furthermore, the enzymic hydrolysis of isoflavone glucosides in soymilk was negatively affected by supplementation with D-glucose and L-cysteine (Chapter 4.0), even though the growth of bifidobacteria was enhanced between 12 and 48 h of incubation compared to plain soymilk (Tables 3.2 and 4.1).

The objectives of this study were to examine the α-galactosidase activity of four strains of *Bifidobacterium* and quantify the oligosaccharide, aldehyde and organic acid levels in soymilk made from SPI590, with and without additional D-glucose and L-cysteine, before and after fermentation by bifidobacteria.

5.2 MATERIALS AND METHODS

5.2.1 Bacteria

Pure cultures of BP20099, BB536, BL1941 and BB12 were obtained from the culture collections mentioned in section 3.2.1. Purity of cultures was checked using Gram staining and each strain was stored under the conditions described in section 3.2.1. These strains of *Bifidobacterium* were selected based on their saccharolytic enzyme activity (Table 3.1).

5.2.2 Bacterial growth media

Rehydrated MRS broth and MRS agar (de Mann *et al.* 1960) each containing additional 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine-HCl were prepared as described in section 3.2.2.

5.2.3 Manufacture of plain and supplemented soymilk

Plain and supplemented soymilks were prepared by reconstituting SPI590 as described in sections 3.2.3 and 4.2.3, respectively. Supplemented soymilk contained additional 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine.

5.2.4 Assay for α-galactosidase activity and the effects of sugar addition on enzyme production

α-Galactosidase activity of BB12, BB536, BL1941 and BP20099 grown in MRS broth, and MRS broth containing additional 1% (w/v) D-glucose (MRS-gluc) or 1% (w/v) D-raffinose (MRS-raf) was determined. Strains were firstly activated in each of MRS, MRS-gluc and MRS-raf broth by transferring successively on 3 occasions (5% v/v inoculum level) with incubation at 37 °C for 20 h. Ten millilitres of activated culture was inoculated in triplicate into 200 mL of each broth and incubated at 37 °C for 48 h. Fifty-millilitre samples were withdrawn aseptically from each broth at 12, 24 and 48 h of incubation and stored at 4°C. Cells were harvested, washed and crude intracellular enzyme extracted as described in section 3.2.4. α-Galactosidase activity was assayed by determining the rate of hydrolysis of the substrate p-nitrophenyl-α-D-galactopyranoside (pNPαG) as described in section 3.2.4. One unit

of enzyme activity was defined as the amount of enzyme required to release 1 μmol of p-nitrophenol from the substrate pNPαG per mL per min under assay conditions (Scalabrini *et al.* 1998), expressed as units of enzyme per mg of protein. The protein content of the enzyme extract was determined by a modified version of the Lowry protein assay (Lowry *et al.* 1951) as described by Rosenberg (1996). p-Nitrophenol and bovine albumin (5 % w/v in 0.7 % w/v sodium chloride) were used to prepare standard linear curves in the enzyme and protein assays, respectively, as described in section 3.2.4. D-raffinose, pNPαG, p-nitrophenol and bovine albumin were purchased from Sigma.

5.2.5 Fermentation of plain and supplemented soymilk by bifidobacteria

Each strain of *Bifidobacterium* was activated prior to the inoculation of plain and supplemented soymilk as described in section 3.2.5. Fermentation of plain and supplemented soymilk and the collection of samples at 0, 12, 24, 36 and 48 h of incubation were then carried out as described in section 3.2.5 and 4.2.4. Samples of 100 mL collected and used for the enumeration of viable *Bifidobacterium* populations and isoflavone analyses in plain and supplemented soymilk were also analysed for their oligosaccharide, organic acid and aldehyde content. Measurements of pH were also taken at each incubation time interval prior to storing the soymilk samples at –20°C.

5.2.6 Analytical standards and reagents

Standards of D-raffinose (pentahydrate), stachyose (trihydrate), D (–)-lactic acid, L (+)-lactic acid and hexanal were obtained from Sigma. D-Glucose, acetic acid, pentanal, sulphuric acid and nitric acid were obtained from Merck Pty. Ltd. (Kilsyth, Victoria, Australia). HPLC grade acetonitrile was obtained from Labscan Analytical Sciences (Bangkok, Thailand).

5.2.7 Extraction and HPLC analysis of raffinose, stachyose and glucose

The extraction of raffinose, stachyose and glucose from non-fermented and fermented soymilk was performed using a modified version of a method developed by Mondy & Kirwan (1993). One millilitre

of 5% (v/v) acetic acid, 29 mL of ultra-pure distilled water and 40 mL of acetonitrile were added to a 30 mL aliquot of soymilk and allowed to stand at room temperature for 30 min. The sample was then passed through a GF/A (glass microfibre) filter paper (Whatman) followed by a IC-Ag solid phase extraction syringe (particle size of 45 to 150 μm) (Alltech Associates Pty. Ltd, Baulkham Hills, NSW, Australia) attached to an Alltech poly-pure filter (0.45 μm polypropylene membrane) after priming with 6 mL of 30% (v/v) acetonitrile. Sugar analyses were carried out on a Waters® high performance liquid chromatograph (Waters Australia Pty. Ltd., Rydalmere, NSW, Australia) comprising a sample processor and injector (model 712), solvent pump (model 515), temperature controlled column compartment (set at 35 °C), and differential refractometer (model 410) connected to a Waters® SAM™ I plain silica amino column (8 mm internal diameter, 100 mm) within a Waters® Radial Compression Module™. The mobile phase consisted of 75% (v/v) acetonitrile filtered through a 0.5 μm FH membrane (Millipore®). A forty-microlitre injection volume was used for samples with an isocratic flow rate of 3 mL per min. The retention times of glucose, raffinose and stachyose were identified using single standards and are shown in Figure 5.1. A mixed standard stock solution comprising 1.151 g of D-raffinose, 1.081 g of stachyose and 2.000 g of D-glucose per 100 mL of mobile phase was diluted to 0.5, 1, 5, 10, 20 and 50 percent of its concentration and 20 μL injected onto the column (multi-level calibration). Mixed sugar standards were analysed using HPLC at the beginning and end of each day of soymilk analyses. Sugar concentrations were calculated back to mg per 100 mL soymilk.

5.2.8 Sample preparation and HPLC analysis of organic acids

Acetic and lactic acids were extracted from fermented soymilk using a method described by Shin *et al.* (2000) with some modifications. Seventy microlitres of concentrated HNO_3 and 5930 μL of 0.009 N H_2SO_4 were added to a 5 mL aliquot of soymilk, vortexed for 20 sec, centrifuged (14000 x g for 10 min) and then filtered (0.20 μm membrane) into a HPLC vial. Chromatographic analyses were carried out on a Varian® 9000 series high performance liquid chromatograph (Varian Pty. Ltd., Mulgrave, Victoria, Australia) with auto-sampler (model 9100), solvent delivery system (model 9010), variable

wavelength UV/VIS detector (model 9050) set at 220 nm, and thermostatically controlled column compartment set at 65 °C. An Aminex HPX-87H ion exclusion column (300 x 7.8 mm) connected to a Micro-Guard® cartridge (Bio Rad Laboratories Pty. Ltd., Hercules, CA, USA) was used to separate acetic acid and L (+)-lactic acid using an isocratic flow rate of 0.6 mL per min of 0.009 N H_2SO_4 filtered through a 0.45 μm membrane (Millipore®) and degassed using a stream of nitrogen. A fifty-microlitre injection volume was used for both samples and standards with the retention time of L (+)-lactic acid and acetic acid shown in Figure 5.2. A mixed standard stock solution of acetic acid (10.5 g per 100 mL) and L (+)-lactic acid (12.2 g per 100 mL) was prepared and diluted to 0.1, 0.5, 1, 3, 5 and 10 percent of its concentration and each were analysed using HPLC at the beginning and end of each day of soymilk analyses. Organic acid levels were calculated back to milligram per 100 mL soymilk.

5.2.9 Analysis of hexanal and pentanal using gas chromatography

The contents of hexanal and pentanal were determined in 5 mL of non-fermented and fermented soymilk with sample preparation previously described by Scalabrini *et al.* (1998). Analyses were carried out on a Varian® 3400x series gas chromatograph equipped with a flame ionization detector set at 220 °C and BP20 (polyethylene glycol) capillary column (12 m x 0.22 mm internal diameter; 0.25 μm film thickness) (SGE International Pty. Ltd., Ringwood, Victoria, Australia). Nitrogen was used as the carrier gas at 400 kPa. The volume of the headspace gas injected was 2 mL with the temperature of the gas chromatograph injector set at 200°C. The column was maintained at 40 °C during the transfer of the headspace components; after 5 min, the column was programmed to increase in temperature by 10 °C per min up to 100 °C. The retention times of pentanal and hexanal were identified using single standards and are shown in Figure 5.3. For multi-level calibration, a mixed standard stock solution containing 0.83 mg of hexanal and 0.81 mg of pentanal per L of ultra-pure distilled water (pH adjusted to 10 to assist dissolution) was diluted to 1, 2.5, 5, 10, 12.5 and 20 percent of its concentration and each were analysed using gas chromatography (GC) at the beginning and end of each day of soymilk

analyses. Concentrations of hexanal and pentanal in soymilk were calculated back to μg per 100 mL soymilk.

5.2.10 Statistical analysis

Growth of each *Bifidobacterium* strain for α-galactosidase assays and for the fermentation of soymilk was performed in triplicate on 2 occasions. Assays for α-galactosidase activity and quantification of sugars, organic acids and aldehydes in soymilk were performed in duplicate and are presented as a mean ± standard error of twelve replicates. Measurements of pH are expressed as a mean ± standard error of six replicates. To find significant differences in α-galactosidase activity, changes in pH, sugar, organic acid and aldehyde composition of soymilks comprising different strains, means were analysed with one-way ANOVA and 95% confidence intervals using Microsoft® Excel StatPro™ as described by Albright *et al.* (1999). ANOVA data with a $P<0.05$ was classified as statistically significant.

5.3 RESULTS AND DISCUSSION

5.3.1 α-Galactosidase activity of bifidobacteria and the effects of glucose and raffinose

Levels of intracellular α-galactosidase produced by BB12, BB536, BL1941 and BP20099 grown in MRS, MRS-gluc and MRS-raf broth for 12, 24 and 48 h at 37°C are shown in Table 5.1. On the whole, the lowest levels of α-galactosidase were produced in MRS broth, which contains 2% (w/v) D-glucose. Only BB12 and BL1941 showed detectable levels of α-galactosidase in MRS broth after 12, 24 and 48 h of incubation. The addition of 1% (w/v) D-glucose in MRS-gluc broth (total of 3% w/v D-glucose) stimulated the production of intracellular α-galactosidase for each of the strains assayed, possibly by providing more favourable conditions for bacterial growth due to the extra carbon source. In MRS-raf broth, 1% (w/v) D-raffinose was included (in addition to 2% w/v D-glucose) because it is a α-galactoside sugar commonly found in soymilk and we wanted to observe whether it enhanced the synthesis of α-galactosidase. Supplementation with D-raffinose significantly increased the production

of α-galactosidase by BB536 and BP20099 ($P<0.05$), with little and no effect on the α-galactosidase activity of BB12 and BL1941, respectively.

As shown in Table 5.1, the highest level of intracellular α-galactosidase was produced by BB536 grown in MRS-raf broth, with significantly higher levels than BB12 at 12, 24 and 48 h of incubation ($P<0.05$). BB536 also produced significantly higher levels of α-galactosidase in MRS-gluc broth at 12 and 24 h of incubation compared to BB12, BL1941 and BP20099 ($P<0.05$), but did not produce detectable levels of enzyme in MRS broth. In an earlier study, β-glucosidase produced by BB536 in MRS broth was also below detectable levels at 12, 24 and 48 h of incubation (Table 3.1). MRS broth typically contains 2% (w/v) glucose, which appears to be insufficient to support enzyme production by BB536. BB536 produced detectable levels of enzyme in the presence of additional 1% (w/v) D-glucose and 1% (w/v) D-raffinose (Tables 3.1 and 5.1), suggesting that this strain requires a carbohydrate source of around 3% (w/v) in MRS broth to stimulate its enzyme activity. In contrast, BL1941 did not show detectable levels of α-galactosidase in the presence of additional 1% (w/v) D-raffinose in MRS broth (Table 5.1). Similarly, BL1941 did not produce detectable levels of β-glucosidase in MRS broth with additional D-raffinose, but produced high levels of the enzyme in the presence of 3% (w/v) glucose (Table 3.1). BL1941 appears to have poor enzymic activity in MRS broth containing a heterogenous carbohydrate source.

In every case except that of BL1941 grown in MRS-gluc broth, the highest intracellular α-galactosidase activities for each strain occurred at 12 and 24 of incubation (Table 5.1). Scalabrini *et al.* (1998) also reported that the highest α-galactosidase activity occurred at 12 and 24 h of incubation for 27 strains of *Bifidobacterium* grown in synthetic medium containing lactose, and this corresponded to their exponential phase of growth. BL1941 maintained its α-galactosidase activity in MRS-gluc broth at 12, 24 and 48 h of incubation, possibly due to its slow growth in this medium; a higher level of α-galactosidase was produced at 48 h than at 12 h of incubation.

5.3.2 Metabolism of oligosaccharides and glucose by bifidobacteria in fermented soymilk

Prior to manufacturing the plain and supplemented soymilks, the raffinose and stachyose level of two different batches of SPI590 were quantified, so as to detect for variation in α-galactosyl oligosaccharide content. There was a significant difference in the concentration of raffinose and stachyose between the two batches ($P<0.05$). The SPI590 with the higher concentration of raffinose (1.7 g per 100 g) and stachyose (2.4 g per 100 g) was used for the manufacture of supplemented soymilk, to examine whether the more favourable growth conditions established by D-glucose and L-cysteine supplementation improved the metabolism of these higher levels of oligosaccharide. The plain soymilk was prepared with a batch of SPI590 that contained 1.4 g of raffinose and 1.9 g of stachyose per 100 g. Concentrations of oligosaccharides and other soluble carbohydrates in SPI are known to vary considerably because of losses occurring during the mild alkali extraction of proteins from insoluble carbohydrates (Liu, 1997). The concentration of glucose was also quantified in the plain and supplemented soymilks as it is preferentially metabolised by bifidobacteria during growth (Scardovi & Trovatelli, 1965).

The concentrations of raffinose, stachyose and glucose in plain and supplemented soymilk, before and after 12, 24, 36 and 48 h of fermentation by *Bifidobacterium* sp., are shown in Tables 5.2 and 5.3, respectively. Supplemented soymilk comprised a significantly greater concentration of raffinose, stachyose and glucose compared to plain soymilk ($P<0.05$); the level of raffinose and stachyose in supplemented soymilk was 14.1 and 18.3 mg higher than in plain soymilk, respectively. Compared to the low levels of raffinose and stachyose found in plain and supplemented soymilk (Tables 5.2 and 5.3, respectively), Scalabrini *et al.* (1998) found higher concentrations of both raffinose and stachyose in non-fermented soymilk made from soybeans, at 530 mg and 250 mg per 100 mL, respectively. In contrast, whole bean soymilk (non-fermented) analysed by Hou *et al.* (2000) contained only 69.6 mg of raffinose per 100 mL but 360.2 mg of stachyose per 100 mL. Variation in sugar composition within

whole bean soymilks is potentially due to both agricultural factors (e.g. genetic variation, climate and soil) and soybean processing (e.g. differences in solids content).

Supplementation of soymilk (made from soybeans) with either D-glucose or L-cysteine was reported to stimulate the growth of *Bifidobacterium* sp. after 24 h of incubation at 37 °C (Kamaly, 1997; Chou & Hou, 2000). In earlier studies performed on plain and supplemented soymilk made from SPI, we found that supplementation of soymilk with D-glucose and L-cysteine improved the growth of BB12, BB536 and BP20099 during 12 and 24 h of incubation (Tables 3.2 and 4.1). In parallel, a greater quantity of glucose, raffinose and stachyose was metabolised by BB12, BB536 and BP20099 in supplemented soymilk after 24 h of incubation (Table 5.3) in comparison to plain soymilk (Table 5.2). The greater and more readily available supply of glucose, raffinose and stachyose in supplemented soymilk may have stimulated the production of α-galactosidase required to hydrolyse raffinose and stachyose, as was observed in our enzyme assays (Table 5.1), consequently improving α-galactoside sugar metabolism and enhancing the growth of these strains over 24 h of incubation. Glucose was the most extensively metabolised by each strain during 0 and 24 h of incubation (Tables 5.2 and 5.3); its hexose structure is exclusively degraded by this genus via the fructose-6-phosphate pathway (Scardovi & Trovatelli, 1965).

The highest levels of raffinose and stachyose in plain (Table 5.2) and supplemented (Table 5.3) soymilk were hydrolysed from 0 to 12 h and 12 to 24 h of fermentation by *Bifidobacterium*. In parallel, BB12, BB536 and BP20099 also showed their highest intracellular α-galactosidase activity at 12 and 24 h of incubation (Table 6.1). Overall, these strains of *Bifidobacterium* metabolised a greater level of stachyose in both plain (Table 5.2) and supplemented (Table 5.3) soymilk after 24 h of incubation, in agreement with Hou *et al.* (2000) who reported that *B. infantis* (CCRC 14633) and *B. longum* (B6) metabolised a higher level of stachyose in soymilk after 24 h. In this study and that of Hou *et al.* (2000), greater levels of stachyose were found in soymilk, potentially being more readily available for hydrolysis than raffinose.

In plain and supplemented soymilk (Table 5.2 and Table 5.3, respectively), BP20099 degraded the highest level of glucose after 24 h of fermentation. The greatest reduction in the concentration of raffinose and stachyose occurred in plain and supplemented soymilks fermented by BB536 and BP20099. In plain soymilk, BB536 metabolised the entire raffinose (55.4 mg per 100 mL) after 24 h of incubation (Table 5.2). BP20099 was more effective than BB536 in the degradation of raffinose in supplemented soymilk ($P>0.05$) (Table 5.3). The entire amount of stachyose in plain soymilk (76.0 mg per 100 mL) was metabolised by BB536 and BP20099 after 24 h of incubation (Table 5.2). In addition to similarities in sugar utilisation, previous studies on the viability of BB536 and BP20099 also showed similar increases in their populations after 12 and 24 h of incubation in plain and supplemented soymilk ($P>0.05$) (Tables 3.2 and 4.1).

After 12 and 24 h of incubation, the lowest reductions in the concentration of glucose, raffinose and stachyose occurred in plain and supplemented soymilk fermented by BL1941 (Tables 5.2 and 5.3). In earlier studies, BL1941 showed the poorest growth characteristics in both plain and supplemented soymilk between 0 and 24 h of incubation compared to BB12, BB536 and BP20099 (Tables 3.2 and 4.1). Plain and supplemented soymilks do not appear to favour the growth of BL1941 and this may be due to their composition of sugars. BL1941 did not show detectable α-galactosidase activity in growth medium containing more than one type of fermentable sugar (Table 5.1). Consequently, this may have limited this strain's capacity to grow in soymilk due to poor utilization of oligosaccharides as substrate.

Over 24 and 48 h of incubation, each strain metabolised a greater amount of glucose, raffinose and stachyose in supplemented soymilk (Table 5.3) compared to plain soymilk (Table 5.2), possibly because of the residual supply of sugar still remaining in supplemented soymilk after 24 h. Our previous work also showed the survival of each strain in supplemented soymilk was better than in plain soymilk between 24 and 48 h of incubation (Tables 3.2 and 4.1).

5.3.3 Production of organic acids in soymilk by bifidobacteria

The levels of L (+)-lactic acid and acetic acid produced by BB12, BB536, BL1941 and BP20099 in plain and supplemented soymilk after 12, 24, 36 and 48 h of incubation at 37°C are shown in Table 5.4, and changes in pH in fermented plain and supplemented soymilk depicted in Figure 5.4a and 5.4b, respectively. We also attempted to quantify D (−)-lactic acid in plain and supplemented soymilk, but found that none of the strains produced this isomer during fermentation. Ishibashi & Shimamura (1993) stated that a unique aspect of bifidobacteria is that all the lactic acid by-product produced during fermentation is in the L (+) form.

For each strain of *Bifidobacterium*, the greatest level of organic acid (total of lactic and acetic acids) was produced in supplemented soymilk after 24 and 48 h of incubation (Table 5.4), possibly because of a significantly greater supply of available glucose ($P<0.05$). As a result, pH reductions in supplemented soymilk fermented by BB12, BB536, BL1941 and BP20099 were greater than those occurring in plain soymilk after 12, 24, 36 and 48 h of incubation (Figures 5.4a and 5.4b). Chou & Hou (2000) also reported that supplementing whole bean soymilk with glucose enhanced acid production by *B. infantis* (CCRC 14633) and *B. longum* (B6). A greater level of acid production in supplemented soymilk by BB12, BB536 and BP20099 (Table 5.4) correlated with their superior growth in this medium compared to plain soymilk after 24 h of incubation (Tables 3.2 and 4.1). In plain soymilk, each strain produced their highest level of lactic and acetic acid in the first 12 h of incubation (Table 5.4), corresponding with the greatest reductions in pH in this medium (Figure 5.4a). According to our previous work, the greatest increase in the viable population of BB12, BB536, BL1941 and BP20099 in plain soymilk also occurred during the first 12 h of incubation (exponential growth phase) (Table 3.2). In supplemented soymilk, only BB536 and BL1941 produced their highest level of lactic and acetic acid in the first 12 h of incubation (Table 5.4), with the greatest reduction in pH also occurring during this period (Figure 5.4b). BB12 and BP20099 produced a greater level of lactic and acetic acid between 24 and 36 h of

incubation in supplemented soymilk (Table 5.4), even though the greatest reduction in pH in supplemented soymilk fermented by these strains occurred during the first 12 h of incubation (Figure 5.4b). This may have been due to the pH level before these incubation periods (that is, before 0 to 12 h of incubation and before 24 to 36 h). At a more neutral pH of 6.7 (at 0 h), the lower amount of organic acid produced by BB12 and BP20099 during the first 12 h of incubation appeared to cause a greater reduction in pH, whereas at a more acidic initial pH of 5.0 to 5.2 (at 24 h), even though a greater amount of organic acid was produced by these strains during 24 and 36 h it had a lesser effect on pH reduction.

It is desirable that fermented products contain low quantities of acetic acid, because of its objectionable 'vinegary' taste. De Vries & Stouthamer (1967) reported that bifidobacteria produce acetic acid and lactic acid in a molar ratio of approximately 1.5, with considerable variation observed between *Bifidobacterium* species (Desjardins *et al.* 1990; Dubey & Mistry, 1996). In agreement, Scalabrini *et al.* (1998) and Hou *et al.* (2000) showed that *Bifidobacterium* sp. generally produced higher quantities of acetic acid in fermented soymilk, with considerable variation in lactic and acetic acid production between different strains. As shown in Table 5.4, plain and supplemented soymilk fermented by BB536 and BL1941 contained greater levels of lactic acid at 12, 24, 36 and 48 h of incubation. The average acetic acid/lactic acid ratio produced by BB536 and BL1941 in plain soymilk was 0.8 and 0.1, respectively. In supplemented soymilk fermented by BB536 and BL1941, the average acetic acid/lactic acid ratios were 0.6 and 0.1, respectively, with the levels of lactic acid significantly greater than those produced in plain soymilk at 12, 24, 36 and 48 h of incubation ($P<0.05$) (Table 5.4). Plain and supplemented soymilk fermented by BP20099 contained equivalent levels of acetic and lactic acid at 12, 24, 36 and 48 h of incubation, with average acetic acid/lactic acid ratios of 1.1 (in plain soymilk) and 1.0 (in supplemented soymilk). Only BB12 produced a higher proportion of acetic acid to lactic acid in plain and supplemented soymilk, with an average acetic acid/lactic acid ratio of 1.6 and 1.2, respectively. According to Table 5.4, supplementing soymilk with 1% (w/v) D-glucose significantly

enhanced the production of lactic acid by BB12 after 24, 36 and 48 h of incubation ($P<0.05$). Overall, BB536 and BL1941 produced the most favourable ratio of acetic acid/lactic acid in fermented soymilk. A higher proportion of lactic acid by-product is preferred to acetic acid as it provides a sweeter acidic taste, which is, generally, more favourable to the palate.

The highest producer of organic acids in both plain and supplemented soymilk was BB536, with BP20099 as the second highest producer under the same conditions (Table 5.4). As shown in Figures 5.4a and 5.4b, BB536 and BP20099 showed similar patterns in the reduction of pH in plain and supplemented soymilk after 12, 24, 36 and 48 h of fermentation. Furthermore, both BB536 and BP20099 metabolised the greatest levels of sugar during fermentation (Tables 5.2 and 5.3) and showed the highest increases in viable population in plain and supplemented soymilk (Tables 3.2 and 4.1). In contrast, BL1941 produced the lowest concentration of organic acids in plain and supplemented soymilk after 12, 24, 36 and 48 h of fermentation (Table 5.4), correlating with the smallest reductions in pH in both soymilks (Figures 5.4a and 5.4b).

5.3.4 Change in aldehyde content of soymilk during fermentation by bifidobacteria

Differences in hexanal and pentanal content existed between the two different batches of SPI590 used to manufacture plain and supplemented soymilk. The SPI590 used to prepare the supplemented soymilk, which comprised a higher level of oligosaccharide (section 5.3.2), also contained a higher level of pentanal (240 μg per 100 g) and hexanal (646 μg per 100 g), approximately 5 and 7 times the content found in the batch of SPI590 used to prepare plain soymilk, respectively. Variation in the levels of hexanal and pentanal in SPI may be mainly influenced by the extent of formation of these volatiles in defatted soybean flour, which is subsequently used in the preparation of SPI. Fujimaki *et al.* (1965) reported that a large quantity of hexanal and some lesser amounts of other volatile components were formed in defatted soybean flour by simple autoxidation of lipids remaining in the defatted soybean and via hydroperoxidation catalysed by lipoxygenases.

Concentrations of hexanal and pentanal in plain and supplemented soymilk before and after incubation with BB12, BB536, BL1941 and BP20099 for 12, 24, 36 and 48 h at 37 °C are shown in Table 5.5. Both plain and supplemented soymilk contained significantly greater levels of hexanal to pentanal ($P<0.05$), roughly 2 to 3 times the level. Likewise, Desai *et al.* (2002) and Scalabrini *et al.* (1998) found 1.4 and 1.9 times the level of hexanal to pentanal in commercial soymilk, respectively. Fermentation of plain and supplemented soymilk by each strain of *Bifidobacterium* decreased the levels of hexanal and pentanal. For BL1941, BB536 and BP20099, the most extensive metabolism of hexanal and pentanal occurred in the first 12 h of incubation, correlating with the exponential growth phase of the latter two strains mentioned. BB12 metabolised a greater concentration of hexanal and pentanal between 12 and 36 h of incubation. Overall, a higher amount of hexanal was degraded in plain and supplemented soymilk by *Bifidobacterium* sp. over 48 h of fermentation compared to pentanal, in agreement with Scalabrini *et al.* (1998) and Desai *et al.* (2002). The mechanism of utilisation of hexanal and pentanal by *Bifidobacterium* is not completely understood. Murti *et al.* (1992) suggested that hexanal might be transformed into hexanoic acid by bifidobacteria in soy yoghurt. Bifidobacteria possess complex enzyme systems, which may be responsible for catalyzing the oxidation of aldehydes into carboxylic acids. The better metabolism of hexanal compared to pentanal by *Bifidobacterium* may be because of their respective availability in soymilk made from SPI. A greater level of hexanal may be free or bound weakly on the surface of proteins, making it more available to biotransformation by bifidobacteria. Whereas, most of the pentanal in soymilk may be bound tightly inside proteins, inhibiting its biotransformation by bifidobacteria. According to Inouye *et al.* (2002), hexanal found in SPI could be classified as two types, one type that is free or bound weakly to proteins and the other which is tightly bound inside proteins.

In plain soymilk, BB536 and BP20099 metabolised the entire hexanal and pentanal after 12 h of fermentation. Reductions in hexanal and pentanal content occurring in plain soymilk fermented by

BB12 and BL1941 were significantly smaller than those by BB536 and BP20099 ($P<0.05$). Even though supplementation of soymilk with D-glucose and L-cysteine enhanced the metabolism of hexanal and pentanal by BB12 and BL1941 ($P<0.05$), BB536 and BP20099 still metabolised a significantly greater level of hexanal and pentanal in supplemented soymilk compared to these strains ($P<0.05$).

5.4 CONCLUSIONS

For three of the four strains of *Bifidobacterium*, the addition of D-raffinose in MRS broth stimulated the production of α-galactosidase, with BB536 and BP20099 being the highest producers of the enzyme. Each strain of *Bifidobacterium* metabolised the oligosaccharides and aldehydes in soymilk made from SPI590 to varying degrees. Supplementation of soymilk with D-glucose and L-cysteine enhanced the degradation of raffinose and stachyose by each strain of *Bifidobacterium*, and of hexanal and pentanal by BB12 and BL1941. Due to their high level of α-galactosidase production during growth, BB536 and BP20099 metabolised the greatest concentrations of raffinose and stachyose in soymilk. Raffinose and stachyose were not detected in plain and supplemented soymilk after 48 h of fermentation by BB536. BB536 and BP20099 also metabolised the greatest quantities of hexanal and pentanal during fermentation of soymilk, with non-detectable levels in plain soymilk after only 12 h of incubation. The extent of sugar metabolism during growth of *Bifidobacterium* correlated with the levels of L (+)-lactic acid and acetic acid produced and the consequent reductions in pH occurring during fermentation. BB536 was the highest producer of organic acids in both plain and supplemented soymilk, producing a greater level of L (+)-lactic acid compared to acetic acid. Nevertheless, despite its superior ability to metabolise oligosaccharides and aldehydes and produce a desirable acetic acid/L (+)-lactic acid ratio, BB536 was not selected for the next stage of research because it did not exhibit the highest β-glucosidase activity nor did it hydrolyse the greatest concentration of isoflavone glucosides.

Table 5.1 α-Galactosidase activity (units of enzyme per mg protein[1]) of *Bifidobacterium* sp. incubated for 12, 24 and 48 h at 37°C (mean ± standard error; n = 12)

Growth Medium	Incubation Time (h)	BB12	BB536	BL1941	BP20099
MRS broth	12	0.016±0.002^{b}	ND	0.021±0.001^{a}	ND
	24	0.017±0.001^{b}	ND	0.020±0.001^{a}	ND
	48	0.001±0.001^{b}	ND	0.014±0.001^{a}	ND
MRS-gluc broth	12	0.236±0.004^{d}	0.728±0.007^{a}	0.332±0.005^{c}	0.644±0.013^{b}
	24	0.253±0.002^{d}	0.609±0.010^{a}	0.370±0.013^{c}	0.506±0.006^{b}
	48	0.055±0.001^{b}	0.348±0.006^{a}	0.343±0.003^{a}	0.335±0.004^{a}
MRS-raf broth	12	0.554±0.001^{b}	6.423±0.043^{a}	ND	6.044±0.022^{a}
	24	1.037±0.020^{b}	5.375±0.222^{a}	ND	5.088±0.025^{a}
	48	0.234±0.029^{c}	1.640±0.028^{a}	ND	0.421±0.063^{b}

BB12: *B. animalis*; BB536: *B. longum*; BL1941: *B. longum*; BP20099: *B. pseudolongum*.

MRS-gluc: MRS broth containing additional 1% (w/v) D-glucose; MRS-raf: MRS broth containing additional 1% (w/v) raffinose.

ND: No activity detected in cell-free enzyme extract after 30 min incubation.

Means in the same row with different superscript are significantly different ($P<0.05$).

[1]One unit of enzyme was defined as the amount of enzyme that released 1 μmol of p-nitrophenol from pNPαG per mL per min under assay conditions.

Table 5.2 Concentration of glucose, raffinose and stachyose (mg per 100 mL) in plain[1] soymilk fermented by *Bifidobacterium* sp. for 12, 24, 36 and 48 h at 37°C (mean ± standard error; n = 12)

Sugar	**Change in sugar concentrations between intervals[2]**								
***Bifidobacterium* sp.**	**0 h[3]**		**12 h**		**24 h**		**36 h**		**48 h**
Glucose									
BB12	120.3±0.6	-81.4^{b}	38.9±1.4	-15.5^{cd}	23.4±0.5	-2.1^{c}	21.3±0.6	$+0.3^{a}$	21.6±0.5
BB536	120.3±0.6	-104.0^{c}	16.3±0.3	-0.7^{a}	15.6±0.5	-0.7^{bc}	14.9±0.4	-0.7^{a}	14.2±0.3
BL1941	120.3±0.6	-69.6^{a}	50.7±1.1	-3.5^{b}	47.2±0.9	$+3.3^{a}$	50.5±1.5	-0.4^{a}	50.1±1.6
BP20099	120.3±0.6	-104.2^{d}	16.1±1.0	-16.1^{d}	ND		ND		ND
Raffinose									
BB12	55.4±0.7	-12.8^{b}	42.6±0.9	-10.5^{b}	32.1±0.9	-2.6^{a}	29.5±0.8	-1.1^{a}	28.4±0.8
BB536	55.4±0.7	-35.9^{d}	19.5±0.8	-19.5^{c}	ND		ND		ND
BL1941	55.4±0.7	-4.6^{a}	50.8±1.0	-1.9^{a}	48.9±0.2	-0.6^{a}	48.3±0.6	-0.8^{a}	47.5±1.1
BP20099	55.4±0.7	-21.8^{c}	33.6±1.5	-26.1^{d}	7.5±0.6	-7.5^{b}	ND		ND
Stachyose									
BB12	76.0±1.2	-8.9^{a}	67.1±0.6	-13.3^{b}	53.8±0.7	-2.2^{a}	51.6±0.7	-2.9^{b}	48.7±0.7
BB536	76.0±1.2	-29.5^{b}	46.5±0.3	-46.5^{d}	ND		ND		ND
BL1941	76.0±1.2	-9.1^{a}	66.9±0.78	-0.8^{a}	66.1±0.3	-1.5^{a}	64.6±0.4	-1.1^{a}	63.5±0.5
BP20099	76.0±1.2	-37.4^{c}	38.6±0.1	-38.6^{c}	ND		ND		ND

BB12: *B. animalis*; BB536: *B. longum*; BL1941: *B. longum*; BP20099: *B. pseudolongum*.

ND: Not detected in a 30 mL aliquot of soymilk used to extract sugars with a sample injection volume of 40 µL.

One-way ANOVA of means arranged as a column of 4 values. Means in the same column with different superscript are significantly different ($P<0.05$).

[1]Soymilk comprising 4% (w/v) soy protein isolate 590.

[2]Mean of the difference in sugar concentrations between incubation times.

[3]Concentration of glucose, raffinose and stachyose in non-fermented plain soymilk (prior to inoculation with activated culture of *Bifidobacterium* sp.).

Table 5.3 Concentration of glucose, raffinose and stachyose (mg per 100 mL) in supplemented[1] soymilk fermented by *Bifidobacterium* sp. for 12, 24, 36 and 48 h at 37°C (mean ± standard error; n = 12)

Sugar	Change in sugar concentrations between intervals[2]								
***Bifidobacterium* sp.**	**0 h[3]**	**12 h**		**24 h**		**36 h**		**48 h**	
Glucose									
BB12	1254.7±15.9	−325.1^{ab}	929.6±55.4	−127.3^{a}	802.3±76.2	−60.7^{a}	741.6±17.7	−18.0^{ab}	723.6±21.0
BB536	1254.7±15.9	−401.1^{bc}	853.6±45.8	−97.2^{a}	756.4±34.7	−94.8^{a}	661.6±41.5	−153.3^{b}	508.3±21.5
BL1941	1254.7±15.9	−202.9^{a}	1051.8±43.0	−56.0^{a}	995.8±15.7	−5.3^{a}	990.5±11.8	−21.6^{ab}	968.9±18.8
BP20099	1254.7±15.9	−506.6^{c}	748.1±22.3	−75.3^{a}	672.8±95.9	−169.9^{a}	502.9±27.6	+6.2^{a}	509.1±18.9
Raffinose									
BB12	69.5±2.0	−22.0^{b}	47.5±3.6	−13.4^{ab}	34.1±3.3	−12.5^{b}	21.6±0.4	−0.8^{a}	20.8±0.5
BB536	69.5±2.0	−42.5^{d}	27.0±1.6	−11.7^{ab}	15.3±3.1	−8.0^{ab}	7.3±4.0	−7.3^{a}	ND
BL1941	69.5±2.0	−1.0^{a}	68.5±4.6	−1.4^{a}	67.1±4.0	+0.2^{a}	67.3±0.8	−3.3^{a}	64.0±1.0
BP20099	69.5±2.0	−36.6^{cd}	32.9±1.1	−20.4^{b}	12.5±1.3	−8.3^{ab}	4.2±3.5	−4.2^{a}	ND
Stachyose									
BB12	94.3±3.5	−15.6^{ab}	78.7±7.5	−19.1^{ab}	59.6±6.1	−13.3^{a}	46.3±0.2	−0.4^{a}	45.9±0.2
BB536	94.3±3.5	−37.1^{b}	57.2±5.3	−42.5^{b}	14.7±2.3	−14.7^{a}	ND		ND
BL1941	94.3±3.5	−6.0^{a}	88.3±6.5	−2.1^{a}	86.2±7.8	−5.8^{a}	80.4±0.4	−0.5^{a}	79.9±0.50
BP20099	94.3±3.5	−28.6^{ab}	65.7±7.3	−12.5^{ab}	53.2±7.0	−10.7^{a}	42.5±6.4	−2.9^{a}	39.6±0.15

BB12: *B. animalis*; BB536: *B. longum*; BL1941: *B. longum*; BP20099: *B. pseudolongum*.

ND: Not detected in a 30 mL aliquot of soymilk used to extract sugars with a sample injection volume of 40 µL.

One-way ANOVA of means arranged as a column of 4 values. Means in the same column with different superscript are significantly different ($P<0.05$).

[1]Soymilk prepared using 4% (w/v) soy protein isolate 590 and supplemented with 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine.

[2]Mean of the difference in sugar concentrations between incubation times.

[3]Concentration of glucose, raffinose and stachyose in non-fermented supplemented soymilk (prior to inoculation with activated culture of *Bifidobacterium* sp.).

Table 5.4 Concentration of L (+)-lactic acid and acetic acid (mg per 100 mL) in soymilks fermented by *Bifidobacterium* sp. for 12, 24, 36 and 48 h at 37°C (mean ± standard error; n = 12)

Organic acid	**Fermentation interval**			
***Bifidobacterium* sp. (soymilk)**	**12 h**	**24 h**	**36 h**	**48 h**
L (+)-Lactic acid				
BB12 (P)	27.1 ± 1.6^{fg}	26.6 ± 1.3^{h}	28.3 ± 1.6^{h}	27.8 ± 2.2^{h}
BB536 (P)	57.1 ± 1.0^{bcde}	57.9 ± 5.3^{efg}	65.2 ± 2.1^{ef}	62.8 ± 2.2^{efg}
BL1941 (P)	25.9 ± 6.6^{g}	34.8 ± 5.0^{gh}	44.9 ± 1.6^{gh}	44.1 ± 1.5^{gh}
BP20099 (P)	42.5 ± 2.1^{defg}	45.3 ± 2.4^{fgh}	47.5 ± 1.3^{fgh}	47.4 ± 1.6^{fgh}
BB12 (S)	38.8 ± 4.1^{efg}	61.9 ± 6.3^{def}	125.0 ± 7.9^{cd}	135.4 ± 10.6^{cd}
BB536 (S)	80.3 ± 4.8^{a}	135.8 ± 3.5^{a}	178.1 ± 4.0^{a}	200.9 ± 5.2^{a}
BL1941 (S)	75.0 ± 4.1^{ab}	89.7 ± 5.3^{bc}	114.6 ± 2.8^{d}	128.1 ± 11.2^{d}
BP20099 (S)	50.1 ± 1.2^{cde}	89.1 ± 2.0^{c}	155.3 ± 3.4^{b}	151.2 ± 4.8^{bcd}
Acetic acid				
BB12 (P)	37.8 ± 0.7^{f}	44.2 ± 1.1^{d}	47.0 ± 0.4^{f}	48.1 ± 0.8^{de}
BB536 (P)	41.8 ± 2.1^{ef}	44.6 ± 3.7^{cd}	50.1 ± 1.0^{ef}	46.7 ± 3.0^{e}
BL1941 (P)	2.3 ± 0.4^{h}	3.3 ± 0.5^{f}	4.3 ± 0.3^{h}	6.3 ± 1.0^{g}
BP20099 (P)	42.1 ± 1.9^{def}	49.9 ± 2.8^{bcd}	51.6 ± 1.1^{def}	51.3 ± 1.3^{cde}
BB12 (S)	50.6 ± 2.6^{bcde}	77.9 ± 6.1^{a}	132.6 ± 1.3^{b}	140.3 ± 4.5^{a}
BB536 (S)	49.5 ± 2.7^{cde}	80.0 ± 2.3^{a}	105.6 ± 2.3^{c}	118.9 ± 3.2^{b}
BL1941 (S)	8.3 ± 2.0^{gh}	5.5 ± 0.1^{ef}	6.6 ± 0.5^{gh}	14.6 ± 3.6^{fg}
BP20099 (S)	61.0 ± 1.5^{a}	86.9 ± 2.6^{a}	147.1 ± 3.6^{a}	141.6 ± 4.5^{a}

BB12: *B. animalis*; BB536: *B. longum*; BL1941: *B. longum*; BP20099: *B. pseudolongum*.
P (plain): Soymilk comprising 4% (w/v) soy protein isolate 590; S (supplemented): Soymilk prepared using 4% (w/v) soy protein isolate 590 and supplemented with 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine.
One-way ANOVA of means arranged as a column of 8 values. Means in the same column with different superscript are significantly different ($P<0.05$).

Table 5.5 Concentration of hexanal and pentanal (μg per 100 mL) in soymilks fermented by *Bifidobacterium* sp. for 12, 24, 36 and 48 h at 37°C (mean ± standard error; n = 12)

Aldehyde	Change in aldehyde concentrations between intervals[1]								
***Bifidobacterium* sp. (soymilk)**	**0 h[2]**		**12 h**		**24 h**		**36 h**		**48 h**
Hexanal									
BB12 (P)	3.60±0.12	-0.13^{a}	3.47±0.12	-0.37^{a}	3.10±0.14	-0.49^{cd}	2.61±0.02	-0.28^{a}	2.33±0.05
BB536 (P)	3.60±0.12	-3.60^{bcde}	ND		ND		ND		ND
BL1941 (P)	3.60±0.12	-1.20^{a}	2.40±0.10	-0.98^{a}	1.42±0.02	-0.29^{bcd}	1.13±0.01	-0.03^{a}	1.10±0.01
BP20099 (P)	3.60±0.12	-3.60^{cde}	ND		ND		ND		ND
BB12 (S)	25.83±0.22	-5.33^{e}	20.50±0.66	-6.00^{d}	14.50±0.14	-2.18^{e}	12.32±0.48	$+0.10^{a}$	12.42±0.33
BB536 (S)	25.83±0.22	-11.46^{fg}	14.37±0.34	-1.15^{ab}	13.22±0.13	-1.83^{d}	11.39±0.06	$+0.29^{a}$	11.68±0.04
BL1941 (S)	25.83±0.22	-4.60^{de}	21.23±0.44	-3.43^{b}	17.80±0.19	-2.24^{f}	15.56±0.23	-4.02^{b}	11.54±0.26
BP20099 (S)	25.83±0.22	-12.51^{g}	13.32±0.24	-5.01^{c}	8.31±0.16	$+0.13^{a}$	8.44±0.45	-0.39^{a}	8.05±0.10
Pentanal									
BB12 (P)	1.87±0.05	-0.59^{a}	1.28±0.01		1.28±0.01	-0.20^{a}	1.08±0.01	-0.02^{a}	1.06±0.01
BB536 (P)	1.87±0.05	-1.87^{cd}	ND		ND		ND		ND
BL1941 (P)	1.87±0.05	-0.24^{a}	1.63±0.02	-0.40^{a}	1.23±0.01	-0.22^{a}	1.01±0.01	-0.01^{a}	1.00±0.01
BP20099 (P)	1.87±0.05	-1.87^{d}	ND		ND		ND		ND
BB12 (S)	9.60±0.04	-1.27^{b}	8.33±0.05	-2.22^{d}	6.11±0.06	-2.38^{c}	3.73±0.08	-0.17^{a}	3.56±0.08
BB536 (S)	9.60±0.04	-4.24^{f}	5.36±0.15	-1.55^{bc}	3.81±0.03	-0.34^{ab}	3.47±0.05	-0.12^{a}	3.35±0.04
BL1941 (S)	9.60±0.04	-3.22^{e}	6.38±0.15	-0.07^{a}	6.31±0.09	-0.68^{b}	5.63±0.07	$+0.02^{a}$	5.65±0.06
BP20099 (S)	9.60±0.04	-5.2^{g}	4.40±0.13	-1.67^{c}	2.73±0.14	-0.09^{a}	2.64±0.14	$+0.03^{a}$	2.67±0.10

BB12: *B. animalis*; BB536: *B. longum*; BL1941: *B. longum*; BP20099: *B. pseudolongum*.

P (plain): Soymilk comprising 4% (w/v) soy protein isolate 590; S (supplemented): Soymilk prepared using 4% (w/v) soy protein isolate 590 and supplemented with 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine.

ND: Not detected in a 5 mL aliquot of soymilk using a headspace gas injection volume of 2 mL.

One-way ANOVA of means arranged as a column of 8 values. Means in the same column with different superscript are significantly different ($P<0.05$).

[1]Mean of the difference in aldehyde concentrations between incubation times.

[2]Concentration of hexanal and pentanal in non-fermented soymilk (prior to inoculation with activated culture of *Bifidobacterium* sp.).

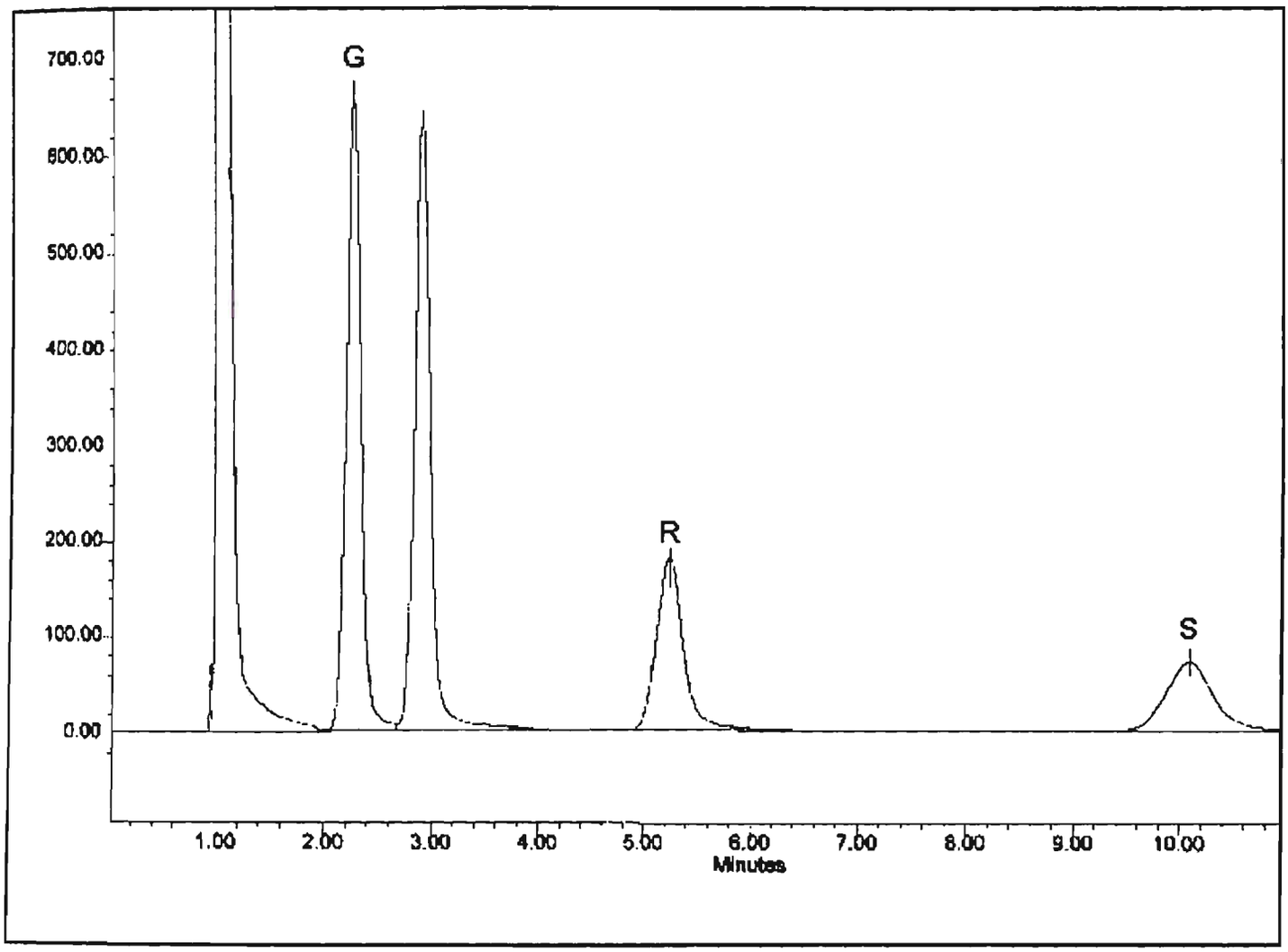

Figure 5.1 HPLC chromatogram showing the approximate retention times of oligosaccharides and glucose found in plain and supplemented non-fermented soymilk; (G) glucose, 2.2 min; (R) raffinose, 5.2 min; (S) stachyose, 10.0 min.

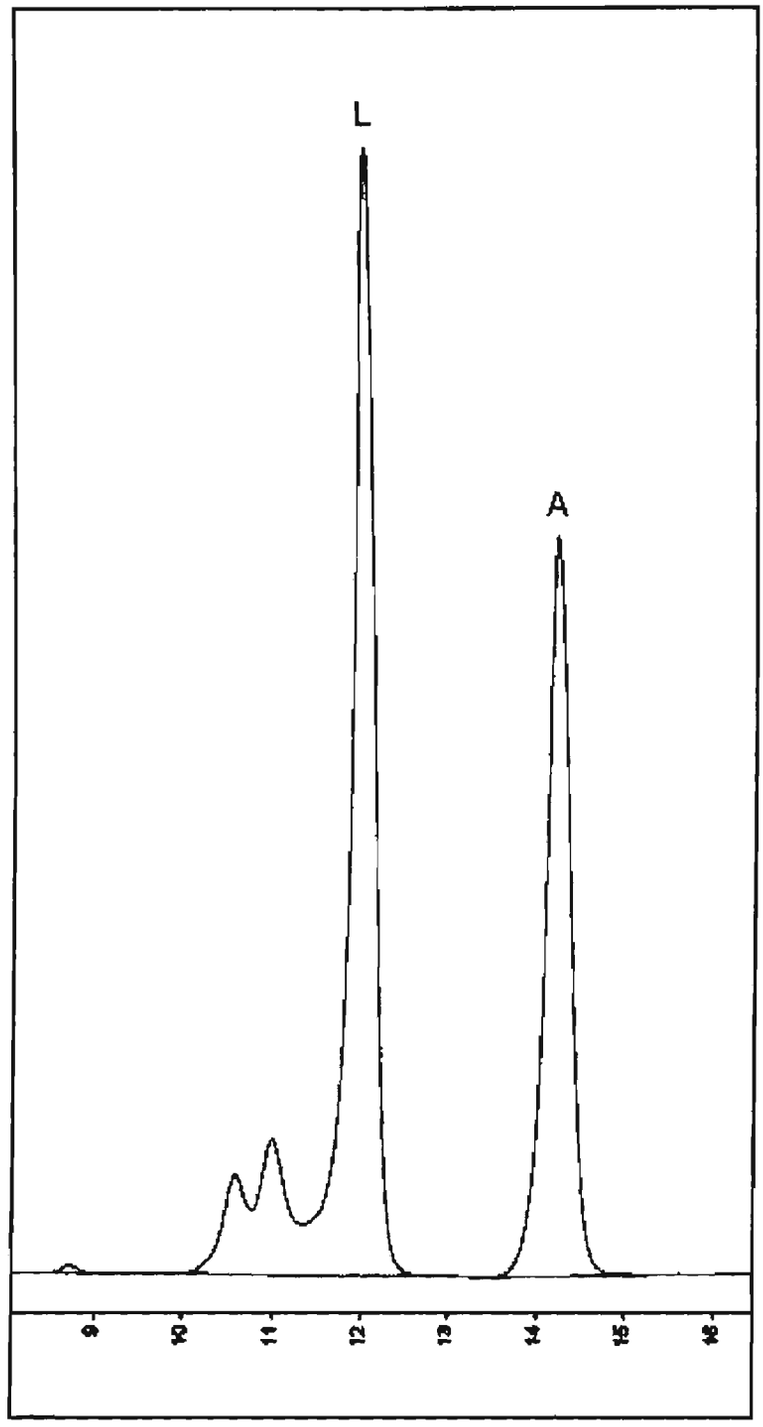

Figure 5.2 HPLC chromatogram showing the approximate retention times of organic acids found in plain and supplemented soymilk fermented by bifidobacteria; (L) L(+)-lactic acid, 12.1 min; (A) acetic acid, 14.3 min.

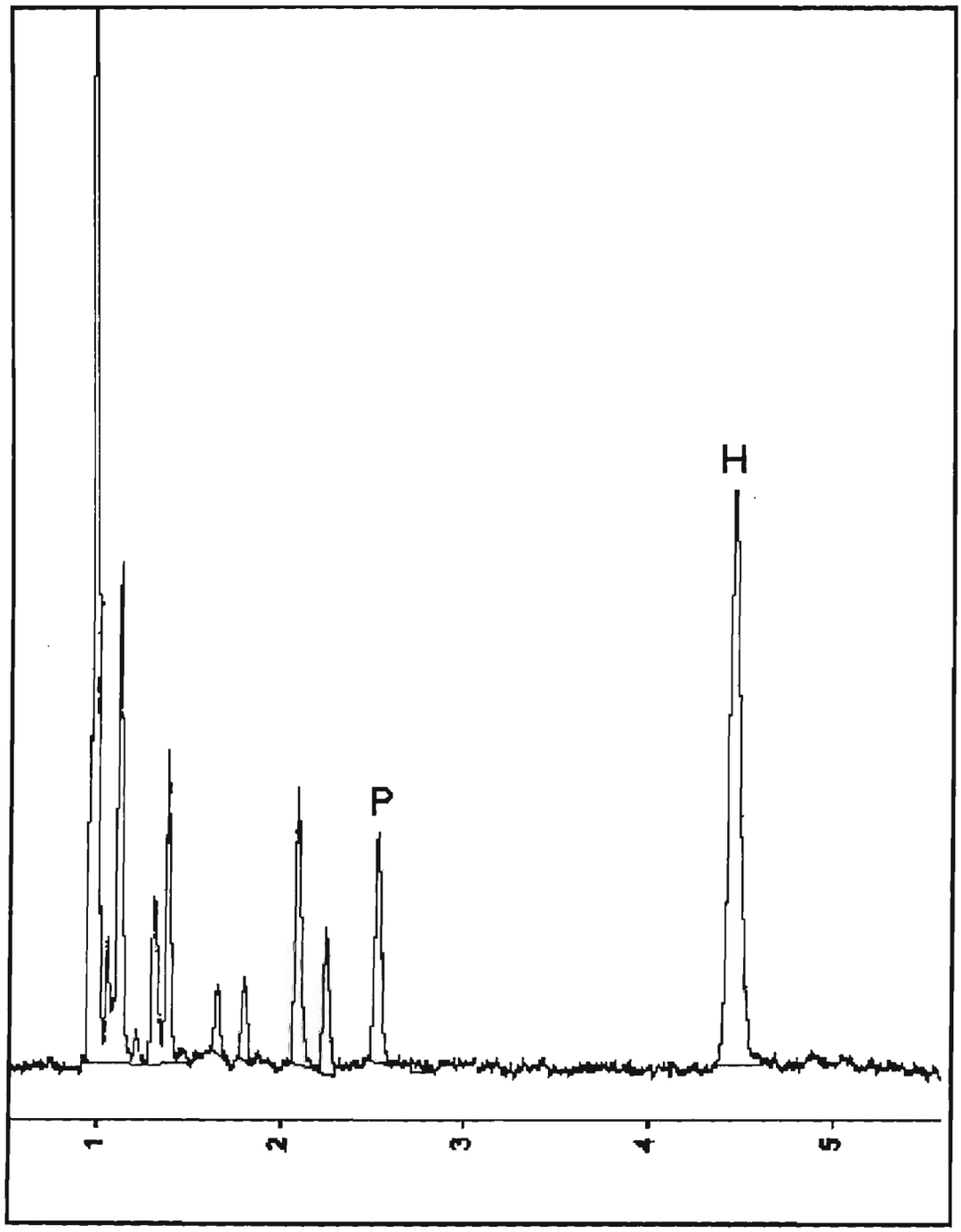

Figure 5.3 GC chromatogram showing the approximate retention times of aldehydes found in plain and supplemented non-fermented soymilk; (P) pentanal, 2.5 min; (H) hexanal, 4.5 min.

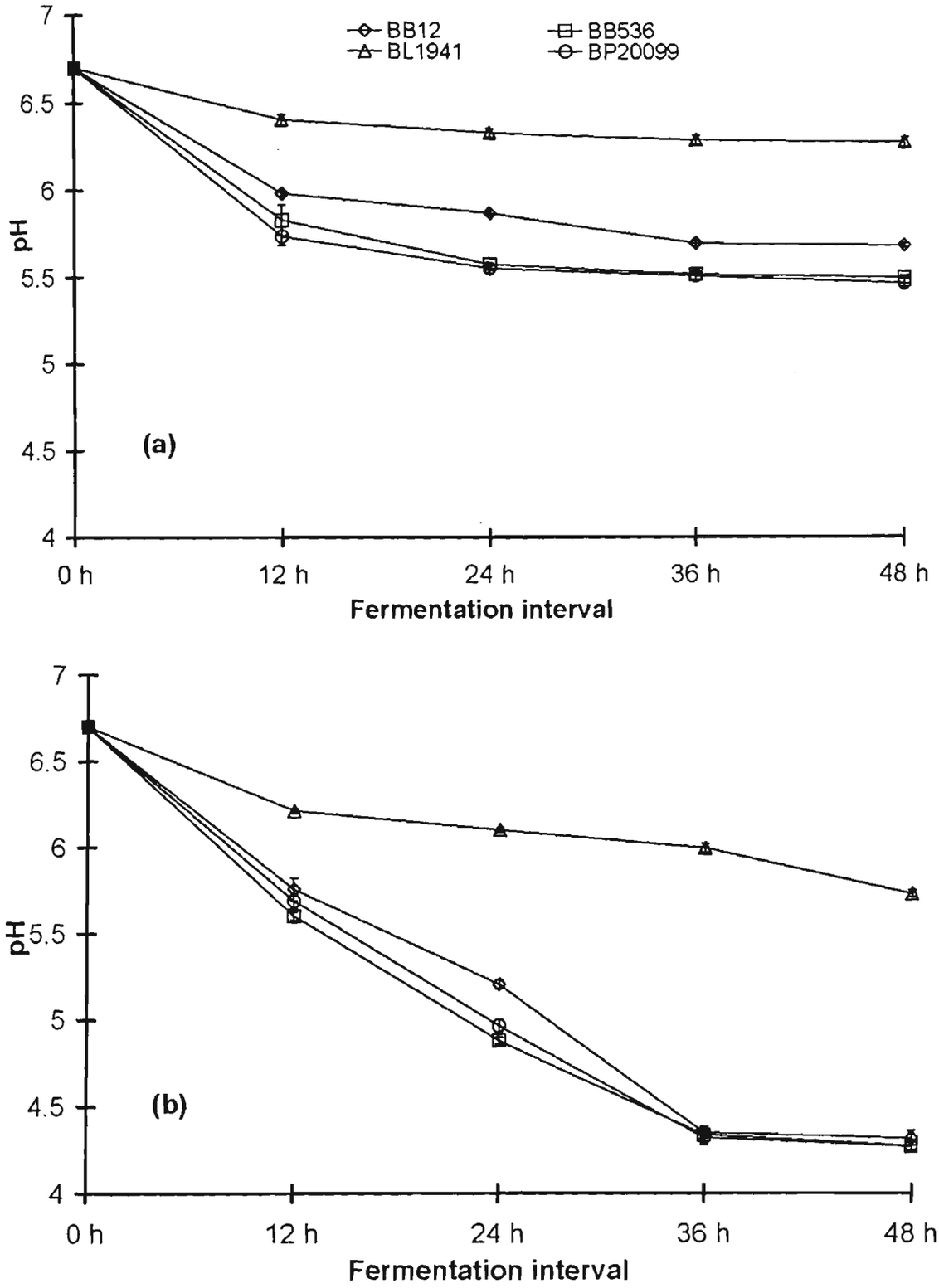

Figure 5.4 The pH levels of soymilk fermented by BB12 (*B. animalis*), BL1941 (*B. longum*), BB536 (*B. longum*) and BP20099 (*B. pseudolongum*) for 12, 24, 36 and 48 h at 37 °C; (a) plain soymilk (4% w/v soy protein isolate); (b) supplemented soymilk (4% w/v soy protein isolate with additional 1% w/v D-glucose and 0.05% w/v L-cysteine). Data points and error bars represent a mean ± standard error of six replicates.

6.0 Development of an Isoflavone Aglycone-Enriched Fermented Soymilk Using Soy Germ, Soy Protein Isolate and Bifidobacteria

6.1 INTRODUCTION

To simplify production and improve the flavour profile of soymilk suitable for Western consumers, modern methods utilise defatted soy material like SPI instead of whole soybeans. However, soymilk prepared by reconstituting SPI has a reduced level of biologically active isoflavones. In the preparation of SPI, significant losses in isoflavone (of up to 53%) occur during the mild alkali extraction of proteins and carbohydrates (Wang & Murphy, 1996).

SG or hypocotyl is the part of the soybean that contains the highest concentration of isoflavone, 5 to 6 times higher than in an equivalent mass of cotyledon (Kudou *et al.* 1991). SG is not used in the manufacture of soymilk due to its content of insoluble carbohydrates (resulting in sedimentation after reconstitution) and low proportion in the whole soybean (2% dry weight) making it difficult to separate from other soybean parts commercially (Schryver, 2002). Nevertheless, because of its high concentration of isoflavone, SG could be used in combination with SPI in soymilk manufacture to enhance the content of isoflavone.

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Of the total concentration of isoflavone in soymilk greater than 90% exists in a glucosidic form, as reported in section 4.3.2. Izumi *et al.* (2000) found that aglycone forms were absorbed faster and in greater amounts than their glucosides in humans. Furthermore, Setchell *et al.* (2002*a*) reported that isoflavone glucosides were not absorbed intact across the enterocyte of healthy adults, and their bioavailability required initial hydrolysis of the sugar moiety by intestinal β-glucosidases. This suggests that consuming isoflavone aglycone-rich soy foods may be more effective in preventing chronic disease. In Chapters 3.0 and 4.0, it was reported that BB12 hydrolysed a significantly greater concentration of isoflavone glucoside into aglycone compared to BP20099, BB536 and BL1941 when grown in soymilk ($P<0.05$), increasing the concentration of aglycone in plain soymilk from 8% to 66% of total isoflavone after 24 h of fermentation (Table 3.6). Hence, BB12 was selected from these strains of *Bifidobacterium* for the development of an isoflavone aglycone-enriched fermented soymilk based on its high β-glucosidase activity and superior ability to biotransform isoflavones in soymilk (Chapter 3.0). BB12 did not metabolise flatus-causing oligosaccharides or objectionable aldehyde compounds in fermented soymilk as effectively as BP20099 and BB536 (Chapter 5.0), but these attributes were only of secondary importance considering the major objectives of this research.

The objective of this study was: 1) to examine the biotransformation of isoflavone glucoside into aglycone in soymilk made from SPI and SG using BB12 during fermentation; and 2) to assess the stability of isoflavones and viability of BB12 over 14 days of refrigerated storage.

6.2 MATERIALS AND METHODS

6.2.1 Ingredients

Pure culture of BB12 was obtained from the culture collection mentioned in section 3.2.1 and stored under the conditions described in section 3.2.1. Supro® FXP-H-159 soy protein isolate (SPI159) was obtained from Protein Technologies International which, according to manufacturer specifications, contained a minimum of 90 g of protein, 4 g of fat and ≤ 4 g of soluble carbohydrates per 100 g.

IsoLife® SG (granular size of 45 μm) was obtained from Soy Health Pty. Ltd. (Sydney, NSW, Australia) and comprised a macronutrient composition of 40 g of protein, 16 g of fat, 25 g of carbohydrate and 3 g of fibre per 100 g.

6.2.2 Bacterial growth media

Rehydrated MRS agar (de Mann *et al.* 1960) containing additional 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine-HCl was prepared as described in section 3.2.2. For the activation of BB12, 40 g of SPI159 and 10 g of food-grade glucose powder (Prahran Health Foods, Prahran, Victoria, Australia) per 1 L of filtered drinking water was reconstituted as described in section 3.2.3. The pH of the soymilk was 6.8.

6.2.3 Manufacture of soymilk

Three soymilk formulations were manufactured, each containing a different ratio of SPI159 to SG (total of 4% w/v); calculated based upon the concentration of isoflavone in each soy ingredient according to manufacturer specifications on SPI159 and a previous study on this brand of SG by Song *et al.* (1998). SPI159 and SG at ratios of 9:1 (SPI/SG9:1), 6:4 (SPI/SG6:4) and 3:7 (SPI/SG3:7) were used to manufacture soymilks containing approximately 20, 40 and 60 mg isoflavone per 100 mL, respectively. In addition, soymilk controls containing 4% (w/v) SPI159 (SPI-soymilk) and 4% (w/v) SG (SG-soymilk) were also prepared. For SPI/SG9:1, 180 g of SPI159 and 20 g of SG were reconstituted in 5 L of filtered drinking water heated to 40°C, followed by heating with stirring at 70°C for 30 min. The soymilk was then dispensed into glass bottles in 1 and 4 L quantities and autoclaved at 121°C for 15 min. One-hundred millilitre aliquots from the 1 L bottle of non-fermented soymilk were obtained under aseptic conditions and freeze dried as described in section 3.2.5 for the extraction of isoflavone and analysis using HPLC. The remaining 4 L of sterile soymilk was cooled to 40 °C prior to inoculation with active culture of BB12. SPI/SG6:4, SPI/SG3:7, SPI-soymilk and SG-soymilk were also

manufactured in the same manner using 120 g of SPI159 to 80 g of SG, 60 g of SPI159 to 140 g of SG, 200 g of SPI159 and 200 g of SG, respectively, per 5 L of filtered drinking water.

6.2.4 Fermentation of soymilk by bifidobacteria

Pure culture of BB12 was activated by 3 successive transfers using an inoculum level of 5% (v/v) with incubation at 37°C for 20 h. For soymilk fermentation studies, a 5% (v/v) inoculum of active BB12 was transferred to 4 L of sterile SPI/SG9:1, SPI/SG6:4, SPI/SG3:7, SPI-soymilk and SG-soymilk. Two hundred millilitres of inoculated soymilk was dispensed into 12 sterile 200 mL high-density polyethylene (HDPE) bottles (Cospak Pty. Ltd., Braeside, Victoria, Australia) under aseptic conditions using a 100 mL Trubor® bottle top dispenser. Bottles of inoculated soymilk were incubated at 37°C for 24 h followed by refrigerated storage for 14 days at 4°C. Two bottles of soymilk were randomly taken from the batch at 0, 12 and 24 h of incubation and at 1, 7 and 14 days of refrigerated storage. A sample of 100 mL was withdrawn aseptically from each bottle for enumeration of viable BB12 populations, pH measurement and the remainder stored at –20°C. Frozen samples of soymilk were freeze-dried as described in section 3.2.5 for the extraction of isoflavone and analysis using HPLC.

6.2.5 Enumeration of bifidobacteria in fermented soymilk

The pour plate method was used for the enumeration of viable populations of BB12. The growth medium used for enumeration and incubation conditions are mentioned in section 3.2.6.

6.2.6 Extraction and HPLC analysis of isoflavones

The extraction of isoflavone aglycone and glucoside isomers from 2.00 g of freeze-dried SPI-soymilk, 1.00 g of freeze-dried SPI/SG9:1 or 0.50 g of either freeze-dried SPI/SG6:4, SPI/SG3:7 or SG-soymilk was performed in duplicate using the method described by Setchell *et al.* (2001). In addition, isoflavone isomers were extracted from 4 replicates of SPI159 and SG using 2.00 g and 0.50 g of powder, respectively. The preparation of mixed and single isoflavone standards used in HPLC analyses is

described in section 3.2.8. Chromatographic analyses were carried out on a Hewlett Packard® 1100 series high performance liquid chromatograph (Agilent Technologies) with diode array UV/VIS detector (set at a wavelength of 260 nm) connected to a Keystone Scientific® ODS-C18 (250 x 4.6 mm internal diameter; 5 μm) reversed-phase column (section 3.2.9). All reagents used in the extraction of isoflavone and HPLC analyses were filtered through a 0.5 μm membrane (section 3.2.9). Gradient elution was used to isolate the isoflavones for detection as described in section 3.2.10. Quantification of isoflavone malonyl-, acetyl-, β-glucoside and aglycone isomers is also described in section 3.2.10. Isoflavone concentrations were calculated back to wet basis (expressed as mg isoflavone per 100 mL of soymilk).

6.2.7 Evaluating the precision of the HPLC method

Intra- and inter-assay %COV for the concentration of each isoflavone isomer in SG powder (0.50 g) was evaluated according to the procedure described in section 3.2.11. The intra- and inter-assay %COV for each isoflavone isomer concentration in SG was as follows: daidzein, 10.0, 8.0; genistein, 3.1, 6.9; glycitein, 2.5, 6.0; daidzin, 2.8, 5.3; genistin, 2.7, 5.8; glycitin, 2.8, 5.3; acetyldaidzin, 1.8, 4.2; acetylgenistin, 2.4, 4.5; acetylglycitin, 2.0, 15.4; malonyldaidzin, 7.4, 7.5; malonylgenistin, 5.1, 6.9; and malonylglycitin, 20.7, 7.3, respectively. The intra- and inter-assay %COV for the total isoflavone concentration found in SG was 2.7 and 5.8, respectively, similar to the values found in earlier analyses on SPI590 (section 3.2.11).

6.2.8 Statistical analysis

Manufacture, fermentation and refrigerated storage of each type of soymilk was performed on two occasions with concentrations of isoflavone isomers and viable counts of BB12 expressed as a mean ± standard error of eight replicates. Measurements of pH are expressed as a mean ± standard error of four replicates. Quantified levels of isoflavone isomers in SPI159 and SG powder are presented as a mean ± standard error of four replicates. To find significant differences between isoflavone isomer

concentrations, viable counts of BB12 and pH measurements, means were analysed with ANOVA and 95% confidence intervals using Microsoft® Excel Stat Pro™ as described by Albright *et al.* (1999). ANOVA data with a $P<0.05$ was classified as statistically significant.

6.3 RESULTS AND DISCUSSION

6.3.1 Isoflavone composition of SPI and SG

The concentration of total and individual isoflavone isomers found in SPI159 and SG are presented in Table 6.1. SG comprised a total isoflavone concentration of 19.398 mg per gram, and this was significantly higher than the 1.375 mg per gram found in SPI159 ($P<0.05$). Song *et al.* (1998) reported that this brand of SG contained a total isoflavone level of 23.2 mg per gram. According to manufacturer specifications on SG, the concentration of isoflavone is expected to vary, mainly due to agricultural factors (genetic variation, climate and soil), with an average of 20 ± 3 mg per gram. The level of isoflavone found in SPI159 was only 40% of the concentration specified by the manufacturer; in this case, variation is primarily due to losses during the processing (mild alkali extraction) of defatted soybean meal into protein isolate (Wang & Murphy, 1996). SPI159 was selected for this study because it contained, according to manufacturer specifications, an isoflavone level of approximately 3.4 mg per gram, roughly 2.8 times the concentration of isoflavone in SPI590, which was used to prepare soymilk in earlier fermentation studies (Chapters 3.0, 4.0 and 5.0). However, it was found that SPI159 and SPI590 comprised equivalent levels of isoflavone, in the range of 1.2 to 1.4 mg per gram.

In the SG, the highest concentration of isoflavone isomers were found in a β-glucosidic configuration, representing 66% of the total isoflavone level (Table 6.1). SG only comprised a total of 0.875 mg per gram of aglycone (5% of total). SPI159 also contained the highest portion of its isoflavone amount in a β-glucoside form (78% of total), with 0.137 mg per gram of aglycone structures (Table 6.1), a higher percentage than that found in SG.

The concentration of glycitein and its glucosidic conjugates in SG was 11.694 mg per gram (60% of total), higher than both daidzein and genistein and their respective glucosides (Table 6.1). SG powder is a concentrated source of isoflavone naturally found in the soybean's hypocotyl, the only part of the bean that contains glycitein and its glucosides at an amount higher than both daidzein and genistein and their glucosides (Kudou *et al.* 1991). Unlike SG, SPI159 comprised a greater concentration of genistein and its glucosidic conjugates, at 0.892 mg per gram (65% of total) (Table 6.1). The manufacture of SPI usually involves the whole soybean of which the cotyledon constitutes the highest proportion of the seed and comprises 80 to 90% of the isoflavone concentration. Genistein and its glucosides are typically found at highest concentration in the soybean cotyledon (Kudou *et al.* 1991), with SPI having a similar isoflavone composition.

6.3.2 Viability of BB12 in soymilk during fermentation and refrigerated storage

To effectively modulate intestinal microbial balance it is essential that BB12 be alive and in sufficient numbers in the fermented soymilk at the time of consumption. It has been suggested that bifidobacteria be present in cultured milk/soymilk to a minimum level of 6 $\log_{10}$ CFU per mL in order to provide a therapeutic effect (Gomes & Malcata, 1999). Hence, the aim was to attain a viable level of at least 8 $\log_{10}$ CFU per mL in each soymilk fermented by BB12. In an earlier study (Chapter 3.0), BB12 increased in population by 0.94 $\log_{10}$ CFU per mL in plain soymilk after 24 h of incubation, with a decrease in its population observed between 24 and 48 h of incubation (Table 3.2). Consequently, a fermentation period of 24 h with an inoculum level of at least 7 $\log_{10}$ CFU per mL of activated culture was used. In the same study (Chapter 3.0), there was very little bioconversion of isoflavone glucosides into aglycone after 24 h of soymilk fermentation by BB12 ($P>0.05$), so limiting the incubation period to 24 h would have little effect on the extent of enrichment of aglycone forms. Glucose was not added to any of the soymilks made from SPI159 and SG so as to potentially enhance the hydrolysis of isoflavone glucosides into aglycone, according to Chapter 4.0. The concentration of isoflavone aglycone in plain soymilk fermented by BB12 (Table 3.6) was higher after 24 h of incubation compared to that in

glucose-supplemented soymilk (Table 4.2), coinciding with a greater reduction in the concentration of isoflavone β-glucosides in plain soymilk. From a sensory viewpoint, the supplementation of soymilk with glucose and a fermentation period in excess of 24 h may have adversely affected the flavour of the final product. In previous work (Chapter 5.0), soymilk supplemented with glucose and fermented by BB12 for up to 36 and 48 h contained significantly greater levels of acetic acid than plain soymilk fermented for 24 h ($P<0.05$) (Table 5.4). A high amount of acetic acid is undesirable in fermented milk because of its objectionable 'vinegary' taste. Lastly, the initial pH of each soymilk was not adjusted with acidulants or alkaline agents, as the objective was to use these soymilks for separate human dietary intervention studies (see Chapters 7.0, 8.0 and 9.0).

Changes in the pH and populations of BB12 in SPI/SG9:1, SPI/SG6:4, SPI/SG3:7, SPI-soymilk and SG-soymilk before and after incubation at 37 °C are shown in Tables 6.2 and 6.3, respectively. The initial pH of SPI/SG9:1, SPI/SG6:4, SPI/SG3:7, SPI-soymilk and SG-soymilk was 6.87, 6.57, 6.43, 6.94 and 6.05, respectively (Table 6.2). The initial optimum pH range for BB12 is between 6.6 and 6.8 (according to manufacturer specifications). After 12 h of incubation, the increase in population of BB12 grown in SPI/SG9:1 and SPI/SG6:4 was significantly higher than the increase occurring in SPI-soymilk and SG-soymilk ($P<0.05$) (Table 6.3). The poorer growth of BB12 in SG-soymilk may have been due to its initial pH level, below the optimum range for growth. However, the growth of BB12 in SG-soymilk was like that in SPI-soymilk ($P>0.05$) (Table 6.3), with the initial pH of SPI-soymilk similar to that of SPI/SG9:1 at approximately 6.9 (Table 6.2). In this case, the addition of 1 part SG in SPI/SG9:1 may have established a more favourable growth environment for BB12 compared to SPI-soymilk due to the higher proportion of oligosaccharides provided by the SG. Soybean oligosaccharides (raffinose and stachyose) are found at higher concentrations in SG than SPI and are effectively metabolised by *Bifidobacterium* sp. during growth in soymilk (Chou & Hou, 2000; Hou *et al.* 2000). In SPI/SG3:7, the viable count of BB12 increased from 7.51 to 8.44 $\log_{10}$ CFU per mL, and this was significantly different to the increase occurring in SPI/SG6:4 ($P<0.05$) (Table 6.3). Growth of BB12 in SPI/SG9:1

was not significantly different to that occurring in SPI/SG6:4 and SPI/SG3:7 ($P>0.05$) (Table 6.3). Hence, a difference in the level of growth of BB12 between soymilks was potentially due to nutrient availability, especially fermentable oligosaccharides, influenced by the ratio of SPI and SG. There was no clear association between an initial pH outside the optimum range of 6.6 to 6.8 and poorer bacterial growth. Nevertheless, the lower levels of growth of BB12 in SPI/SG3:7, SPI-soymilk and SG-soymilk may have been due to the diversion of energy from anabolic processes to pH homeostasis (Holt *et al.* 1994).

In each of the soymilks, the growth of BB12 between 0 and 12 h of incubation caused the greatest reduction in pH (Table 6.2). There was no significant difference in the reduction of pH in SG-soymilk compared to SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7 after 12 h ($P>0.05$), but the pH drop in SPI-soymilk was significantly different to each soymilk containing SG ($P<0.05$). The presence of SG in SPI/SG9:1, SPI/SG6:4, SPI/SG3:7 and SG-soymilk may have enhanced the acid production of BB12 due to the greater level of oligosaccharide substrate. SG powder is a rich source of oligosaccharides and fibre at approximately 15 g per 100 gram (Schryver, 2002). However, it is most likely that with increasing protein content the buffering capacity of the soymilk was enhanced. As shown in Table 6.3, the growth of BB12 was similar in both SPI-soymilk and SG-soymilk after 12 h of incubation, presumably producing similar levels of acid in each medium, but the pH drop in SG-soymilk was significantly greater (Table 6.2); SPI-soymilk had considerably higher protein content. SPI159 used to prepare SPI-soymilk contained 90 g of protein per 100 g whereas SG comprised 40 g of protein per 100 g.

Growth of BB12 in each of SPI/SG9:1, SPI/SG6:4, SPI/SG3:7, SPI-soymilk and SG-soymilk occurred between 0 and 12 h of incubation (Table 6.3), but it is unlikely that an exponential growth phase occurred during this period due the high initial inoculum level. Between 12 and 24 h of incubation, there was very little or no growth of BB12 in soymilk (Table 6.3), most likely due to the accumulation of organic acids. According to Holt *et al.* (1994), *Bifidobacterium* sp. cannot grow in an environment

where the pH is below 4.5. The pH of SPI/SG6:4, SPI/SG3:7 and SG-soymilk after 12 h of incubation was 4.58, 4.52 and 4.28, respectively. In SPI/SG9:1 and SPI-soymilk, the reduction in growth of BB12 between 12 and 24 h may have been due to the diminishing supply of carbohydrates; found at low concentrations in SPI. Acidification caused gelation in SPI/SG6:4 and SPI/SG3:7 after 12 h of fermentation as the pH dropped to the isoelectric point of soy protein (in the range of pH 4.5) (Liu, 1997).

The pH levels and populations of BB12 in SPI/SG9:1, SPI/SG6:4, SPI/SG3:7, SPI-soymilk and SG-soymilk at 1, 7 and 14 days of refrigerated storage (4°C) are also shown in Tables 6.2 and 6.3, respectively. Between intervals of refrigerated storage, there were fluctuations in BB12 populations in each of SPI/SG9:1, SPI/SG6:4, SPI/SG3:7, SPI-soymilk and SG-soymilk. Each of the soymilks contained acceptable viable levels of probiotic bifidobacteria considered to be of therapeutic benefit (Gomes & Malcata, 1999), with no significant difference in the population at the completion of 24 h of fermentation to that found after 14 days of refrigerated storage ($P>0.05$) (Table 6.3). The main factor for the loss of viability of bifidobacteria in fermented milk during refrigerated storage has been attributed to the detrimental effects of accumulated organic acids (Hughes & Hoover, 1995). As shown in Table 6.2, there were no significant reductions in pH in SPI/SG9:1, SPI/SG6:4, SPI/SG3:7, SPI-soymilk and SG-soymilk during refrigerated storage intervals ($P>0.05$).

6.3.3 Enzymic hydrolysis of isoflavone glucosides in fermented SPI-soymilk and SG-soymilk

The concentrations of individual isoflavone isomers in SPI-soymilk and SG-soymilk before and after fermentation by BB12 for 12 and 24 h at 37°C are shown in Table 6.4. The yield of isoflavone in SPI-soymilk (5.835 mg per 100 mL) made from SPI159 was slightly higher than the expected concentration of approximately 5.5 mg per 100 mL; suggesting that the manufacture of SPI-soymilk did not result in any loss of isoflavone. Non-fermented SG-soymilk comprised a total of 66.783 mg isoflavone per 100 mL, with the greatest proportion of isomers found in a β-glucoside form, like that in SPI-soymilk. SG-

soymilk was expected to contain a total isoflavone concentration of around 77 mg per 100 mL. The lower level of isoflavone in SG-soymilk was possibly due to the poor solubility of SG powder, causing inadequate dissolution of those isoflavones present within the insoluble fraction, which is prone to sedimentation. The distribution of individual isoflavone isomer concentrations in non-fermented SPI-soymilk and SG-soymilk was similar to that in SPI and SG powder (Table 6.1), respectively, implying that the soymilk production process had little or no effect on the composition of isoflavone, as discussed previously in section 4.3.2.

After 12 h of fermentation by BB12, the concentration of daidzein and genistein significantly increased in SPI-soymilk and SG-soymilk ($P<0.05$), in parallel with a significant reduction in the concentration of β-glucoside isomers daidzin and genistin ($P<0.05$) (Table 6.4). Although the growth of BB12 was similar in both SPI-soymilk and SG-soymilk after 12 h (Table 6.3), there was a significant difference in the quantity of isoflavone glucosides hydrolysed ($P<0.05$), with 2.186 mg of isoflavone glucoside per 100 mL hydrolysed in SPI-soymilk compared to 7.260 mg per 100 mL in SG-soymilk (Table 6.4). A greater level of β-glucosidase may have been produced by BB12 in SG-soymilk, possibly stimulated by the presence of fibre and the higher levels of isoflavone glucoside substrate, but had no effect on its growth.

The glucosidic conjugates of glycitein showed the lowest levels of hydrolysis in SPI-soymilk and SG-soymilk at 12 h of fermentation. As a result, there were no significant increases in the concentration of glycitein in SPI-soymilk and SG-soymilk after 12 h ($P>0.05$) (Table 6.4). β-Glucosidase produced by BB12 did not seem to favour the hydrolysis of the glucose moiety of malonylglycitin, acetylglycitin and glycitin. This may be due to the chemical structure of glycitein and the positioning of its functional groups. Glycitein possesses a methoxyl ($-OCH_3$) group at position 6 of its A-ring, whereas genistein has a hydroxyl (-OH) group at position 5 and daidzein has neither of these functional groups at these positions. The glucoside derivative is attached at position 7 of the A-ring and its hydrolytic cleavage

may be inhibited by the proximity of the -OCH_3 group. Steric hindrance (Sykes, 1986) may have been occurring, where the sheer bulk of the -OCH_3 group may have been influencing the reactivity of position 7, by impeding the approach of β-glucosidase to the glucosidic bond.

Fermentation of SG-soymilk for 24 h did not significantly change the overall composition of isoflavone aglycones and glucosides ($P>0.05$) (Table 6.4), as the glucosidic conjugates of glycitein were found at highest concentration and poorly hydrolyzed. In addition, the amount of β-glucosidase produced by BB12 during 24 h of fermentation could have been inadequate for hydrolysing the high concentration of glucosidic conjugates of daidzein and genistein present in SG-soymilk. Xie *et al.* (2003) incubated β-glycosidase in 17% (w/v) SG-soymilk (same brand of SG) and found that a higher concentration of enzyme was needed for optimum hydrolysis of isoflavone glucosides compared to reconstituted soy meal, which comprised an isoflavone glucoside level similar to SPI-soymilk.

Between 12 and 24 h of incubation, the growth of BB12 decreased substantially in SPI-soymilk (Table 6.3), but the biotransformation of isoflavone glucosides to aglycone forms continued, with significant increases in the concentration of daidzein and genistein ($P<0.05$) (Table 6.4). Likewise, in an earlier study (Chapter 3.0), the concentration of aglycone forms significantly increased in plain soymilk (made from SPI590) between 12 and 24 h of fermentation by BB12 ($P<0.05$) (Table 3.6) even though its growth significantly reduced during this period (Table 3.2). In the same study (Chapter 3.0), populations of BB12 decreased in plain soymilk between 24 and 48 h of incubation (death phase) (Table 3.2) and this coincided with very little bioconversion of isoflavone glucoside to aglycone (Table 3.6). Considering the results from previous work we do not believe a longer fermentation period of 36 h or 48 h would have enhanced the bioconversion of isoflavone glucosides to aglycone forms in SPI-soymilk. After 24 h of fermentation by BB12, SPI-soymilk made from 4% (w/v) SPI159 comprised a different composition of isoflavones to that found in its non-fermented form, with the aglycone concentration increasing to 45% of total isoflavone (Table 6.4). However, plain soymilk made from 4%

(w/v) SPI590 and fermented by BB12 for 24 h comprised 66% of total isoflavone in an aglycone configuration (Table 3.6), even though the increase in viable population of BB12 was similar in both plain soymilk (Table 3.2) and SPI-soymilk (Table 6.3) after 24 h.

The total concentration of isoflavone in fermented SPI-soymilk and SG-soymilk prior to refrigerated storage at 4°C was 5.150 and 61.017 mg per 100 mL, respectively, and were lower than the concentrations found prior to fermentation ($P>0.05$) (Table 6.4). Losses in total isoflavone concentration after fermentation were possibly caused by the hydrolytic cleavage of the glucose moiety from the isoflavone glucoside isomers, which contribute to the mass of isoflavones when found as glucoside forms. Hydrolysis of isoflavone glucosides increases the portion of biologically active aglycone; hence, a reduction in total isoflavone concentration does not lower the potential health benefit of the fermented soymilk. Refrigerated storage had little or no effect on the total and individual isoflavone isomer concentrations in fermented SPI-soymilk and SG-soymilk after 1, 7 and 14 days at 4°C ($P>0.05$) (Table 6.4) possibly because of the low levels of microbial and β-glucosidase activity at this temperature.

6.3.4 Enrichment of isoflavone aglycone in fermented soymilk comprising both SG and SPI

The level of intake of isoflavone required to provide an optimum health benefit is not known. From epidemiological studies on Asian populations, high consumption of soy-based foods has been associated with lowered risks of breast, prostate and colon cancer (Messina *et al.* 1994; Messina *et al.* 1997), heart disease (Yamori *et al.* 2001) and osteoporosis (Finkel, 1998). Chen *et al.* (1999) and Wakai *et al.* (1999) estimated that Chinese and Japanese populations consume an average of 30 to 40 mg isoflavone per day, expressed as aglycone constituents. In recent studies by Uesugi *et al.* (2002) and Nicholls *et al.* (2002), a dose of 60 mg isoflavone per day positively affected biomarkers of disease risk, including lipid profiles, bone turnover markers and hormonal status. Hence, we formulated SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7 to contain isoflavone levels per 200 mL serving that covered the

daily intake of Chinese and Japanese populations and those considered of physiological benefit according to small-scale clinical studies.

The total and individual isoflavone isomer concentrations found in non-fermented SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7 are presented in Tables 6.5, 6.6 and 6.7, respectively. The total concentration of glucoside isomers was significantly greater than the level of aglycone forms (5 to 6% of total) ($P<0.05$) in non-fermented SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7. Of the aglycone isomers, daidzein was found at highest concentration in SPI/SG9:1 (Table 6.5), whereas glycitein was highest in SPI/SG6:4 (Table 6.6) and SPI/SG3:7 (Table 6.7) due to the greater quantities of SG present. The total isoflavone concentration in non-fermented SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7, linearly related to the proportion of SG, was approximately 2.7, 7.4 and 12.2 times higher than soymilk made from SPI (roughly 5 mg per 100 mL), respectively. From a sensory perspective, a higher amount of isoflavone may adversely affect the flavour of SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7. Huang *et al.* (1981) and Kudou *et al.* (1991) reported that isoflavones contribute to the undesirable bitterness and astringency associated with soy foods.

In an attempt to enrich the concentration of bioactive and bioavailable aglycone forms in SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7, each was fermented by BB12 for 12 and 24 h at 37°C. The most profound change in the composition of isoflavone isomers occurred in SPI/SG9:1, with the total concentration of aglycone significantly increasing to 47% of total isoflavone after 24 h of fermentation ($P<0.05$) (Table 6.5). The total concentration of acetyl- and β-glucoside forms significantly dropped to represent 7% and 45% of total isoflavone ($P<0.05$), respectively (Table 6.5). Changes in isoflavone isomer composition were less apparent in SPI/SG6:4 and SPI/SG3:7. It pronounced that the β-glucosidase levels produced by BB12 during fermentation were insufficient to hydrolyse the high levels of isoflavone glucoside found in both SPI/SG6:4 and SPI/SG3:7. As a result, the level of aglycone in SPI/SG6:4 and SPI/SG3:7

still only represented 26% and 13% of total isoflavone, respectively, after the entire fermentation period.

Of the individual isomers in SPI/SG6:4 (Table 6.6) and SPI/SG3:7 (Table 6.7), daidzein contributed the greatest portion to the total aglycone level after 24 h of fermentation, as the glucosidic conjugates of daidzein appeared to be effectively hydrolysed during fermentation. Genistein constituted the greatest portion of aglycone in fermented SPI/SG9:1 (after 24 h) (Table 6.5); in this case, the glucosidic conjugates of genistein were found at highest concentration prior to fermentation and also appeared to be effectively hydrolysed. In each of SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7 the increase in the concentration of glycitein during fermentation was less than that of genistein and daidzein, similar to that occurring in SPI-soymilk and SG-soymilk (Table 6.4). However, there were some significant reductions in the concentration of glycitin, acetylglycitin and malonylglycitin in SPI/SG6:4 and SPI/SG3:7, potentially caused by enzymic hydrolysis during fermentation, but were accompanied with only a moderate rise in the concentration of glycitein when compared to the increases of daidzein and genistein. Heinonen *et al.* (2002) reported that glycitein undergoes reductive metabolism by intestinal bacteria, transforming the isomer into an equol analogue, 6-Ome-equol. Hence, the glucosidic conjugates of glycitein in SPI/SG6:4 and SPI/SG3:7 may be undergoing enzymic hydrolysis and reductive metabolism by BB12 during fermentation, causing the reduction in the concentration of glucoside isomers but with no apparent increase in its aglycone level.

On the whole, the greatest level of isoflavone glucoside hydrolysis occurred during 0 and 12 h of fermentation, resulting in larger increases in the concentration of aglycone in each of the soymilks (Tables 6.4, 6.5, 6.6 and 6.7). There was a drop in the level of isoflavone biotransformation between 12 and 24 h of fermentation coinciding with the stationary phase of growth for BB12. The level of growth of BB12 was also associated with the extent of isoflavone biotransformation. For example, the greatest increases in the populations of BB12 occurred in SPI/SG9:1 and SPI/SG6:4 after 24 h of incubation

(Table 6.3) and this correlated with the greatest reduction in the level of isoflavone glucoside and highest increase in the concentration of aglycone (Tables 6.5 and 6.6, respectively).

The stability of isoflavone isomers in fermented SPI/SG9:1 (Table 6.5), SPI/SG6:4 (Table 6.6) and SPI/SG3:7 (Table 6.7) was also monitored during refrigerated storage (4°C). The growth of BB12 in each of SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7 decreased during refrigerated storage (Table 6.3), and this possibly repressed the synthesis and activity of β-glucosidase required to hydrolyse isoflavone glucosides. As a result, there were no significant changes in isoflavone isomer composition in SPI/SG9:1 (Table 6.5), SPI/SG6:4 (Table 6.6) and SPI/SG3:7 (Table 6.7) after 1, 7 and 14 days of refrigerated storage ($P>0.05$). Additionally, there were no significant losses in total isoflavone concentration after 14 days of refrigerated storage of SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7 ($P>0.05$). Therefore, refrigerated storage of fermented SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7 maintained both the viability of BB12 and the composition of isoflavones.

6.4 CONCLUSIONS

SG contained approximately 14 times more isoflavone than SPI159; hence, soymilk comprising a greater portion of SG to SPI159 contained a higher isoflavone concentration. Incubation of each soymilk made from a combination of SPI159 and SG increased the populations of BB12 from ~7.5 to at least 8.4 $\log_{10}$ CFU per mL. The greatest increases in populations of BB12 after 12 h of incubation occurred in SPI/SG6:4 and SPI/SG9:1. Enzymic hydrolysis of acetyl- and β-glucoside isoflavone forms during fermentation increased the concentration of bioactive aglycone structures, to varying degrees, in SPI/SG9:1, SPI/SG6:4 and SPI/SG3:7. The highest concentration of aglycone was found in fermented SPI/SG6:4 after 24 h, even though 74% of its isoflavone concentration was still in an isoflavone glucoside configuration. BB12 was more effective in hydrolysing lower levels of isoflavone glucoside, like that found in SPI/SG9:1. During refrigerated storage, there were no losses or changes in the composition of isoflavones in fermented soymilks. In addition, refrigerated storage maintained the

populations of BB12, possibly as a result of low post-acidification indicated by a stable pH. Concomitant ingestion of a high level of bioavailable isoflavone aglycone and probiotic BB12 may modulate intestinal microbial balance and enhance the absorption of isoflavones, augmenting the health benefit of soymilk.

Table 6.1 Concentration of isoflavone isomers (mg per gram) in SG and SPI159 (mean ± standard error; n = 4)

Isoflavone Isomer	SG	SPI159
Daidzein	0.334 ± 0.009^{hijkl}	0.060 ± 0.002^{fg}
Genistein	0.091 ± 0.002^{kl}	0.068 ± 0.001^{efg}
Glycitein	0.450 ± 0.007^{ghij}	0.009 ± 0.001^{ijkl}
Daidzin	2.990 ± 0.045^{c}	0.233 ± 0.002^{b}
Genistin	1.908 ± 0.031^{d}	0.732 ± 0.017^{a}
Glycitin	7.982 ± 0.121^{a}	0.114 ± 0.001^{c}
Malonyldaidzin	0.086 ± 0.002^{l}	0.001 ± 0.0001^{l}
Malonylgenistin	0.247 ± 0.004^{ijkl}	0.010 ± 0.0001^{jkl}
Malonylglycitin	0.193 ± 0.010^{jkl}	0.002 ± 0.0001^{kl}
Acetyldaidzin	1.329 ± 0.028^{e}	0.046 ± 0.002^{gh}
Acetylgenistin	0.719 ± 0.014^{fg}	0.082 ± 0.001^{def}
Acetylglycitin	3.069 ± 0.061^{bc}	0.018 ± 0.001^{hijkl}
Aglycone[1]	0.875 ± 0.015^{A}	0.137 ± 0.002^{B}
β-Glucoside[2]	12.880 ± 0.196^{A}	1.079 ± 0.019^{B}
Malonylglucoside[3]	0.526 ± 0.013^{A}	0.013 ± 0.0002^{B}
Acetylglucoside[4]	5.117 ± 0.101^{A}	0.146 ± 0.003^{B}
Total isoflavone	19.398 ± 0.313^{A}	1.375 ± 0.019^{B}

SG: soy germ; SPI159: soy protein isolate 159.

Means in the same column with different lower-case superscript are significantly different ($P<0.05$). Means in the same row with different upper-case superscript are significantly different ($P<0.05$).

[1]Mean total of daidzein, genistein and glycitein.

[2]Mean total of daidzin, genistin and glycitin.

[3]Mean total of malonyldaidzin, malonylgenistin and malonylglycitin.

[4]Mean total of acetyldaidzin, acetylgenistin and acetylglycitin.

Table 6.2 Changes in pH occurring in SPI/SG9:1, SPI/SG6:4, SPI/SG3:7, SPI-soymilk and SG-soymilk fermented by BB12 for 12 and 24 h at 37°C followed by 1, 7 and 14 days of refrigerated storage[1] at 4°C (mean ± standard error; n = 4)

Soymilk Type	Change in pH between intervals (n = 4)											
	0 h[2]		12 h		24 h		1 d		7 d		14 d	
SPI/SG9:1	6.87±0.10	-1.61^{bc}	5.26±0.01	-0.13^{a}	$5.13±0.04^{A}$	$+0.20^{a}$	$5.33±0.01^{A}$	-0.02^{a}	$5.31±0.06^{A}$	-0.08^{a}	$5.23±0.06^{A}$	
SPI/SG6:4	6.57±0.08	-1.99^{e}	4.58±0.05	-0.20^{a}	$4.38±0.04^{A}$	$+0.26^{a}$	$4.64±0.01^{A}$	-0.09^{a}	$4.55±0.03^{A}$	0.00	$4.55±0.06^{A}$	
SPI/SG3:7	6.43±0.04	-1.91^{de}	4.52±0.02	-0.18^{a}	$4.34±0.04^{A}$	$+0.17^{a}$	$4.51±0.01^{A}$	0.00	$4.51±0.08^{A}$	$+0.06^{a}$	$4.57±0.05^{A}$	
SPI-soymilk	6.94±0.01	-0.81^{a}	6.13±0.01	-0.14^{a}	$5.99±0.05^{A}$	-0.02^{a}	$5.97±0.04^{A}$	-0.06^{a}	$5.91±0.02^{A}$	$+0.04^{a}$	$5.95±0.04^{A}$	
SG-soymilk	6.05±0.01	-1.77^{cde}	4.28±0.01	-0.14^{a}	$4.14±0.01^{A}$	-0.03^{a}	$4.11±0.03_{A}$	$+0.04^{a}$	$4.15±0.02^{A}$	-0.03^{a}	$4.12±0.02^{A}$	

SPI/SG9:1: Soymilk containing 9 parts of soy protein isolate 159 to 1 part of soy germ (total of 4% w/v); SPI/SG6:4: Soymilk containing 6 parts of soy protein isolate 159 to 4 parts of soy germ (total of 4% w/v); SPI/SG3:7: Soymilk containing 3 parts of soy protein isolate 159 to 7 parts of soy germ (total of 4% w/v); SPI-soymilk: Soymilk containing 4% (w/v) soy protein isolate 159; SG-soymilk: Soymilk containing 4% (w/v) soy germ.

BB12: *B. animalis*.

Means in the same column with different lower-case superscript are significantly different ($P<0.05$). Means in the same row with different upper-case superscript are significantly different ($P<0.05$).

[1]Soymilk refrigerated for 14 days after fermentation by BB12 for 24 h.

[2]Non-fermented soymilk inoculated with 5% (v/v) activated culture of BB12.

Table 6.3 Changes in viable count (log_{10} CFU per mL) of BB12 in SPI/SG9:1, SPI/SG6:4, SPI/SG3:7, SPI-soymilk and SG-soymilk incubated for 12 and 24 h at 37°C followed by 1, 7 and 14 days of refrigerated storage[1] at 4°C (mean ± standard error; n = 8)

Soymilk Type	Change in log_{10} CFU per mL between intervals (n = 8)											
	0 h[2]		12 h		24 h		1 d		7 d		14 d	
SPI/SG9:1	7.55±0.04	+1.10^{ab}	8.65±0.06	+0.04^{a}	8.69±0.03^{A}	+0.13^{a}	8.82±0.03	−0.17^{e}	8.65±0.03	+0.17^{a}	8.82±0.02^{A}	
SPI/SG6:4	7.46±0.03	+1.17^{a}	8.63±0.03	+0.11^{a}	8.74±0.03^{A}	−0.20^{b}	8.54±0.01	+0.20^{a}	8.74±0.03	−0.01^{cd}	8.73±0.03^{A}	
SPI/SG3:7	7.51±0.03	+0.93^{bcd}	8.44±0.05	0.00	8.44±0.04^{A}	−0.01^{a}	8.43±0.04	−0.04^{d}	8.39±0.04	+0.16^{abcd}	8.55±0.04^{A}	
SPI-soymilk	7.34±0.01	+0.86^{cd}	8.20±0.02	+0.04^{a}	8.24±0.02^{A}	+0.03^{a}	8.27±0.03	+0.06^{bcd}	8.33±0.02	−0.03^{d}	8.30±0.02^{A}	
SG-soymilk	7.11±0.01	+0.82^{d}	7.93±0.02	0.00	7.93±0.03^{A}	+0.04^{a}	7.97±0.01	−0.07^{cd}	7.90±0.03	+0.02^{bcd}	7.92±0.02^{A}	

SPI/SG9:1: Soymilk containing 9 parts of soy protein isolate 159 to 1 part of soy germ (total of 4% w/v); SPI/SG6:4: Soymilk containing 6 parts of soy protein isolate 159 to 4 parts of soy germ (total of 4% w/v); SPI/SG3:7: Soymilk containing 3 parts of soy protein isolate 159 to 7 parts of soy germ (total of 4% w/v); SPI-soymilk: Soymilk containing 4% (w/v) soy protein isolate 159; SG-soymilk: Soymilk containing 4% (w/v) soy germ.

BB12: *B. animalis*.

Means in the same column with different lower-case superscript are significantly different ($P<0.05$). Means in the same row with different upper-case superscript are significantly different ($P<0.05$).

[1]Soymilk refrigerated for 14 days after fermentation by BB12 for 24 h.

[2]Non-fermented soymilk inoculated with 5% (v/v) activated culture of BB12.

Table 6.4 Concentration of isoflavone isomers (mg per 100 mL) in SPI–soymilk and SG–soymilk inoculated with BB12 and incubated for 12 and 24 h at 37°C (mean ± standard error; n = 8)

Isoflavone Isomer	SPI–soymilk			SG–soymilk		
	0 h[1]	12 h	24 h	0 h[1]	12 h	24 h
Daidzein	0.224±0.010^{c}	0.549±0.046^{b}	0.754±0.063^{a}	1.455±0.027^{c}	2.726±0.033^{b}	3.416±0.061^{a}
Genistein	0.182±0.011^{c}	1.024±0.055^{b}	1.520±0.095^{a}	0.415±0.028^{c}	1.605±0.043^{b}	2.315±0.059^{a}
Glycitein	0.039±0.004^{a}	0.041±0.001^{a}	0.047±0.001^{a}	1.305±0.037^{a}	1.508±0.045^{a}	1.735±0.076^{a}
Daidzin	1.409±0.162^{a}	0.903±0.056^{bc}	0.866±0.047^{c}	17.090±0.262^{a}	14.616±0.284^{bc}	14.400±0.143^{c}
Genistin	2.897±0.132^{a}	1.620±0.103^{bc}	1.283±0.069^{c}	7.861±0.099^{a}	7.203±0.102^{b}	6.836±0.093^{c}
Glycitin	0.462±0.023^{a}	0.395±0.018^{a}	0.449±0.039^{a}	21.488±0.245^{a}	19.779±0.310^{bc}	19.525±0.220^{c}
Malonyldaidzin	0.063±0.006^{a}	0.067±0.006^{a}	0.043±0.003^{b}	0.869±0.034^{a}	0.819±0.050^{a}	0.699±0.070^{a}
Malonylgenistin	0.054±0.003^{a}	0.034±0.002^{a}	0.044±0.011^{a}	1.451±0.021^{a}	1.302±0.022^{bc}	1.256±0.038^{c}
Malonylglycitin	0.009±0.003^{a}	0.005±0.002^{a}	0.004±0.004^{a}	0.391±0.011^{a}	0.385±0.016^{a}	0.343±0.024^{a}
Acetyldaidzin	0.148±0.077^{a}	0.065±0.011^{a}	0.061±0.023^{a}	5.575±0.073^{a}	4.295±0.058^{b}	3.907±0.054^{c}
Acetylgenistin	0.217±0.010^{a}	0.082±0.008^{bc}	0.056±0.011^{c}	2.080±0.361^{a}	1.568±0.052^{ab}	1.203±0.074^{b}
Acetylglycitin	0.130±0.059^{a}	0.033±0.006^{a}	0.023±0.005^{a}	6.801±0.316^{a}	6.381±0.308^{ab}	5.383±0.352^{b}
Total aglycone[2]	0.445±0.024^{c}	1.615±0.084^{b}	2.321±0.152^{a}	3.176±0.088^{c}	5.839±0.106^{b}	7.466±0.187^{a}
Total isoflavone	5.835±0.389^{a}	4.818±0.251^{a}	5.150±0.331^{a}	66.783±0.868^{a}	62.185±0.883^{bc}	61.017±1.109^{c}

SPI-soymilk: Soymilk containing 4% (w/v) soy protein isolate 159; SG-soymilk: Soymilk containing 4% (w/v) soy germ.

BB12: *B. animalis*.

One-way ANOVA of means arranged as a row of three values for SPI-soymilk and SG-soymilk. Means in the same row with different superscript are significantly different ($P<0.05$).

[1]Concentration of isoflavone isomers in non-fermented and non-inoculated soymilk.

[2]Mean total of daidzein, genistein and glycitein.

Table 6.5 Concentration of isoflavone isomers (mg per 100 mL) in SPI/SG9:1 fermented by BB12 for 12 and 24 h at 37°C followed by 1, 7 and 14 days of refrigerated storage[1] at 4°C (mean ± standard error; n = 8)

Isoflavone	Incubation Time			Refrigeration Time		
Isomer	0 h[2]	12 h	24 h	1 d	7 d	14 d
Daidzein	0.341±0.016^{b}	1.927±0.110^{a}	2.164±0.051^{a}	2.194±0.092^{a}	2.094±0.063^{a}	1.875±0.258^{a}
Genistein	0.256±0.009^{b}	2.493±0.156^{a}	2.936±0.057^{a}	2.911±0.100^{a}	2.763±0.075^{a}	2.721±0.208^{a}
Glycitein	0.222±0.007^{b}	0.462±0.021^{a}	0.545±0.011^{a}	0.552±0.021^{a}	0.533±0.015^{a}	0.515±0.045^{a}
Daidzin	2.488±0.067^{a}	1.007±0.106^{bcdef}	0.890±0.107^{cdef}	0.758±0.086^{ef}	0.732±0.116^{f}	0.781±0.237^{def}
Genistin	4.371±0.123^{a}	1.419±0.215^{bcdef}	1.076±0.197^{cdef}	0.799±0.156^{def}	0.799±0.191^{ef}	0.765±0.268^{f}
Glycitin	3.845±0.107^{a}	3.659±0.062^{abc}	3.438±0.148^{abc}	3.329±0.047^{bc}	3.206±0.126^{c}	3.486±0.054^{abc}
Malonyldaidzin	0.038±0.001^{a}	0.043±0.002^{a}	0.036±0.002^{a}	0.037±0.001^{a}	0.038±0.001^{a}	0.042±0.006^{a}
Malonylgenistin	0.154±0.005^{a}	0.039±0.003^{bcdef}	0.037±0.003^{cdef}	0.033±0.002^{ef}	0.031±0.003^{f}	0.036±0.016^{def}
Malonylglycitin	0.081±0.004^{ab}	0.083±0.003^{a}	0.076±0.004^{abc}	0.075±0.001^{abc}	0.068±0.003^{bc}	0.066±0.006^{c}
Acetyldaidzin	0.494±0.016^{a}	0.229±0.013^{bc}	0.182±0.021^{cdef}	0.146±0.013^{ef}	0.147±0.019^{def}	0.142±0.024^{f}
Acetylgenistin	0.490±0.017^{a}	0.078±0.010^{bcd}	0.044±0.006^{cdef}	0.034±0.003^{def}	0.021±0.008^{f}	0.027±0.011^{ef}
Acetylglycitin	0.894±0.032^{a}	0.589±0.025^{bcdef}	0.517±0.023^{cdef}	0.484±0.016^{ef}	0.429±0.022^{f}	0.515±0.029^{def}
Total aglycone[3]	0.819±0.030^{b}	4.882±0.286^{a}	5.645±0.116^{a}	5.657±0.213^{a}	5.390±0.150^{a}	5.111±0.510^{a}
Total isoflavone	13.674±0.375^{a}	12.028±0.203^{bcdef}	11.941±0.512^{cdef}	11.352±0.132^{def}	10.681±0.395^{f}	10.965±0.136^{ef}

SPI/SG9:1: Soymilk containing 9 parts of soy protein isolate 159 to 1 part of soy germ (total of 4% w/v).

BB12: *B. animalis*.

Means in the same row with different superscript are significantly different ($P<0.05$).

[1]Soymilk refrigerated for 14 days after fermentation by BB12 for 24 h.

[2]Concentration of isoflavone isomers in non-fermented and non-inoculated soymilk.

[3]Mean total of daidzein, genistein and glycitein.

Table 6.6 Concentration of isoflavone isomers (mg per 100 mL) in SPI/SG6:4 fermented by BB12 for 12 and 24 h at 37°C followed by 1, 7 and 14 days of refrigerated storage[1] at 4°C (mean ± standard error; n = 8)

Isoflavone Isomer	Incubation Time			Refrigeration Time		
	0 h[2]	12 h	24 h	1 d	7 d	14 d
Daidzein	0.715±0.031^{c}	2.990±0.194^{b}	3.553±0.133^{a}	3.586±0.122^{a}	3.718±0.041^{a}	3.898±0.057^{a}
Genistein	0.292±0.007^{c}	2.055±0.118^{b}	2.799±0.110^{a}	2.901±0.151^{a}	2.957±0.089^{a}	3.015±0.035^{a}
Glycitein	0.835±0.015^{c}	1.161±0.070^{b}	1.449±0.058^{a}	1.492±0.040^{a}	1.510±0.014^{a}	1.535±0.022^{a}
Daidzin	6.608±0.094^{a}	3.146±0.099^{ef}	3.381±0.173^{def}	3.518±0.040^{bcdef}	3.450±0.061^{cdef}	3.142±0.050^{f}
Genistin	5.815±0.068^{a}	2.700±0.130^{bcdef}	2.357±0.151^{cdef}	2.315±0.078^{def}	2.310±0.073^{ef}	2.193±0.099^{f}
Glycitin	15.025±0.233^{a}	12.452±0.464^{def}	12.156±0.573^{f}	12.584±0.144^{bcdef}	12.539±0.080^{cdef}	12.181±0.159^{ef}
Malonyldaidzin	0.145±0.004^{a}	0.112±0.005^{f}	0.119±0.006^{bcdef}	0.116±0.001^{def}	0.117±0.004^{cdef}	0.115±0.004^{ef}
Malonylgenistin	0.487±0.006^{a}	0.271±0.009^{cdef}	0.273±0.012^{cdef}	0.287±0.011^{bcdef}	0.271±0.012^{ef}	0.257±0.008^{f}
Malonylglycitin	0.331±0.015^{a}	0.264±0.011^{def}	0.263±0.015^{ef}	0.274±0.005^{bcdef}	0.265±0.007^{cdef}	0.259±0.008^{f}
Acetyldaidzin	1.703±0.031^{a}	0.905±0.057^{bc}	0.716±0.046^{cdef}	0.685±0.045^{def}	0.626±0.043^{f}	0.652±0.032^{ef}
Acetylgenistin	1.092±0.016^{a}	0.280±0.039^{b}	0.128±0.017^{cdef}	0.115±0.011^{def}	0.111±0.015^{ef}	0.110±0.015^{f}
Acetylglycitin	3.823±0.092^{a}	2.499±0.122^{bcdef}	2.405±0.128^{cdef}	2.401±0.031^{def}	2.345±0.063^{ef}	2.241±0.083^{f}
Total aglycone[3]	1.842±0.042^{c}	6.206±0.373^{b}	7.801±0.293^{a}	7.979±0.308^{a}	8.185±0.128^{a}	8.448±0.078^{a}
Total isoflavone	36.871±0.477^{a}	28.835±1.243^{f}	29.599±1.343^{def}	30.274±0.368^{cdef}	30.319±0.122^{bcdef}	29.598±0.321^{ef}

SPI/SG6:4: Soymilk containing 6 parts of soy protein isolate 159 to 4 parts of soy germ (total of 4% w/v).

BB12: *B. animalis*.

Means in the same row with different superscript are significantly different ($P<0.05$).

[1]Soymilk refrigerated for 14 days after fermentation by BB12 for 24 h.

[2]Concentration of isoflavone isomers in non-fermented and non-inoculated soymilk.

[3]Mean total of daidzein, genistein and glycitein.

Table 6.7 Concentration of isoflavone isomers (mg per 100 mL) in SPI/SG3:7 fermented by BB12 for 12 and 24 h at 37°C followed by 1, 7 and 14 days of refrigerated storage[1] at 4°C (mean ± standard error; n = 8)

Isoflavone	Incubation Time			Refrigeration Time		
Isomer	0 h[2]	12 h	24 h	1 d	7 d	14 d
Daidzein	1.077±0.071^{b}	2.580±0.077^{a}	2.926±0.053^{a}	2.876±0.042^{a}	2.855±0.045^{a}	2.873±0.139^{a}
Genistein	0.354±0.026^{c}	1.408±0.027^{b}	1.764±0.037^{a}	1.728±0.039^{a}	1.781±0.046^{a}	1.729±0.058^{a}
Glycitein	1.539±0.109^{b}	1.720±0.030^{ab}	1.845±0.026^{a}	1.799±0.024^{a}	1.854±0.040^{a}	1.781±0.016^{a}
Daidzin	10.495±0.780^{a}	8.196±0.099^{bcdef}	8.141±0.154^{def}	7.962±0.151^{ef}	8.235±0.057^{cdef}	7.845±0.411^{f}
Genistin	6.951±0.513^{a}	5.507±0.098^{bcdef}	5.172±0.082^{cdef}	5.065±0.068^{ef}	5.159±0.037^{def}	4.431±0.213^{f}
Glycitin	25.990±1.947^{a}	22.544±0.337^{a}	22.725±0.415^{a}	22.005±0.380^{a}	22.785±0.147^{a}	22.858±0.704^{a}
Malonyldaidzin	0.277±0.021^{a}	0.264±0.003^{ab}	0.263±0.010^{ab}	0.246±0.011^{ab}	0.243±0.009^{ab}	0.209±0.016^{b}
Malonylgenistin	0.842±0.064^{a}	0.646±0.009^{cdef}	0.649±0.016^{bcdef}	0.635±0.016^{ef}	0.639±0.008^{def}	0.641±0.031^{f}
Malonylglycitin	0.631±0.064^{a}	0.482±0.015^{abcde}	0.442±0.023^{de}	0.414±0.022^{e}	0.455±0.022^{bcde}	0.452±0.036^{cde}
Acetyldaidzin	3.269±0.243^{a}	2.136±0.066^{bcde}	1.825±0.044^{def}	1.784±0.043^{ef}	1.849±0.037^{cdef}	1.504±0.093^{f}
Acetylgenistin	1.920±0.149^{a}	0.868±0.034^{bc}	0.558±0.021^{cdef}	0.530±0.021^{def}	0.520±0.014^{ef}	0.467±0.032^{f}
Acetylglycitin	7.515±0.560^{a}	5.645±0.157^{bcdef}	5.290±0.099^{cdef}	5.139±0.064^{ef}	5.171±0.054^{def}	4.376±0.245^{f}
Total aglycone[3]	2.970±0.201^{c}	5.708±0.132^{b}	6.535±0.110^{a}	6.403±0.099^{a}	6.490±0.122^{a}	6.383±0.199^{a}
Total isoflavone	60.860±4.516^{a}	51.996±0.875^{ab}	51.601±0.808^{ab}	50.183±0.721^{ab}	51.546±0.312^{ab}	49.166±1.578^{a}

SPI/SG3:7: Soymilk containing 3 parts of soy protein isolate 159 to 7 parts of soy germ (total of 4% w/v).

BB12: *B. animalis*.

Means in the same row with different superscript are significantly different ($P<0.05$).

[1]Soymilk refrigerated for 14 days after fermentation by BB12 for 24 h.

[2]Concentration of isoflavone isomers in non-fermented and non-inoculated soymilk.

[3]Mean total of daidzein, genistein and glycitein.

7.0 Bioavailability of Isoflavone Phytoestrogens in Postmenopausal Women Consuming Soymilk Fermented by Bifidobacteria

7.1 INTRODUCTION

In a recent study, Richelle *et al.* (2002) discovered that the enzymic hydrolysis of isoflavone glucosides into aglycone forms in a soy drink (made from SG) before consumption did not enhance the absorption of isoflavones in postmenopausal women after a single dose; plasma and urine isoflavone pharmacokinetics were similar for both aglycone- and glucoside-rich soy drinks. In contrast, Hutchins *et al.* (1995) and Slavin *et al.* (1998) reported that fermentation of cooked soybeans by *Rhizopus oligosporus* (tempeh) enhanced the bioavailability of daidzein and genistein in men and women over a 9-day feeding period (112 g of tempeh per day) compared to the ingestion of non-fermented cooked soybean pieces under identical conditions. Intestinal bacteria play an important role in the ultimate bioactivities of isoflavones (Hendrich, 2002; Turner *et al.* 2003). To date, studies on the pharmacokinetics of isoflavones in humans have not investigated the effects of fermenting soy foods by bifidobacteria. Bifidobacteria constitute a major part of the natural microflora of the human intestine, with their highest populations in the ileum and colon (Orrhage & Nord, 2000). In Chapters 3.0, 4.0 and 6.0, it was reported that β-glucosidase-producing BB12 hydrolysed the greatest concentration of isoflavone glucosides into bioactive aglycone forms when grown in soymilk. In clinical studies, BB12 has also shown to effectively modulate intestinal microbial balance (Playne, 2002). Hence, the enrichment of isoflavone aglycone in soymilk by fermentation prior to consumption and the modulation of intestinal microflora via the ingestion of viable bifidobacteria may enhance the bioavailability of isoflavones when consumed on a daily basis.

A version of this chapter is forthcoming. Tsangalis D, Wilcox G, Shah NP & Stojanovska L (2004) *Br J Nutr.*

The objective of this study was to investigate the effects of the daily consumption of an isoflavone aglycone-enriched fermented soymilk containing viable populations of BB12 on urinary isoflavone excretion and percentage recovery in postmenopausal women.

7.2 SUBJECTS AND METHODS

7.2.1 Subjects

Sixteen healthy postmenopausal women were recruited from the Melbourne metropolitan area from fifty-two interested volunteers. Each volunteer was interviewed and screened using a health information and food frequency questionnaire (Appendix C) designed to exclude those who had: gastrointestinal disorders; food allergies; an alcohol intake greater than two standard drinks per day; regularly used prescription or non-prescription medication; were on hormone replacement therapy or antibiotics in the past 3 months; or had dietary patterns that were not representative of the general population (e.g. strict vegetarian, consumption of more than two servings of soy food per week). Participants were asked to read a plain language statement outlining the reasons for and requirements of the study, sign a consent form (Appendix D), and then were randomly allocated to one of two groups (eight subjects per group), to consume either fermented or non-fermented soymilk during the supplementation periods. The mean (± standard deviation) age of women in the fermented soymilk (FS) and non-fermented soymilk (NFS) group was 52.5 (± 3.1) and 55.6 (± 5.1) years, respectively. Five women in the FS group and 6 women in the NFS group had not had any menstrual bleeding for at least 12 months; the other recruited women had not menstruated for at least 6 months.

7.2.2 Study design

The study protocol was approved by the Human Research Ethics Committee of Victoria University (Melbourne, Victoria, Australia) and consisted of a randomised, double-blind, crossover design involving three 14-day soymilk supplementation periods separated by two 14-day washout periods. Subjects consumed either fermented or non-fermented soymilk containing three different

concentrations of total isoflavone during each respective 14-day supplementation period. All batches of fermented soymilk contained a consistent population of BB12, at 10^8 viable cells per mL. Two-hundred millilitres of chilled (4°C) fermented soymilk was consumed per day, 100 mL before breakfast and before dinner. Each participant consumed a self-selected diet for the entire study and kept a 14-day weighed food and beverage record (Appendix E) during each supplementation period. Subjects were advised not to alter their diet for the duration of the study and were asked to exclude foods containing soy as an ingredient, chickpeas, lentils, beans (all types), alfalfa, mung bean sprouts, fermented dairy products, probiotic supplements and alcoholic beverages. Daily energy, macronutrient and dietary fibre intake were quantified for each subject using Food Works version 3.01 nutrition software utilising a database of Australian foods (Xyris Software Pty. Ltd., Highgate Hill, Queensland, Australia). Anthropometric measurements of height (m) and bodyweight (kg) were recorded and the body mass index (BMI) of each subject calculated (kg/m^2) before and after each supplementation period.

7.2.3 Collection and handling of urine specimens

Each participant collected four 24-hour pooled urine specimens per soymilk supplementation period; a day before beginning soymilk supplementation (baseline) and on days 4, 13 and 14. Twenty-four hour pooled urine specimens consisted of the second urination on the allocated day and those following and including the first urination on the next day and were passed into 3.2 L sterile plastic bottles (Biocorp Pty. Ltd., Huntingdale, Victoria, Australia) containing 2 g of ascorbic acid (Sigma) as preservative against chemical degradation. Specimens were stored at 4°C or on ice until they were analysed. The total volume of each 24-hour pooled urine specimen was measured and a 100 mL aliquot taken for urinary isoflavone analyses. Prior to storage at –20 °C, 1 mL of 10% (w/v) sodium azide (Labchem Pty. Ltd., Auburn, NSW, Australia) was added to the aliquot as a preservative against microbial spoilage.

7.2.4 Soymilk ingredients

Pure culture of BB12 was obtained from the culture collection mentioned in section 3.2.1 and stored under the conditions described in section 3.2.1. SPI159 and SG were obtained from the suppliers mentioned in section 6.2.1. The macronutrient composition of SPI159 and SG (according to manufacturer specifications) is also described in section 6.2.1. The total concentration of isoflavone in SPI159 and SG was 1.4 and 19.4 mg per gram, respectively (Table 6.1). Vanilla flavour (in propylene glycol) was purchased from Essential Flavours and Ingredients (Rowville, Victoria, Australia).

7.2.5 Manufacture of soymilk

Three soymilk formulations were manufactured, each containing a different ratio of SPI159 to SG (total of 4% w/v soy ingredients); calculated based upon the concentration of isoflavone in each soy ingredient (section 7.2.4). SPI159 and SG at ratios of 9.4:0.6, 8.0:2.0 and 5.2:4.8 were used to manufacture soymilks containing approximately 20 (SPI/SG20), 40 (SPI/SG40) and 80 (SPI/SG80) mg isoflavone per 200 mL serving, respectively. These levels of isoflavone covered the daily intake typically associated with Asian populations, in the range of 30 to 40 mg isoflavone aglycone (Chen *et al.* 1999; Wakai *et al.* 1999). The concentration of isoflavone aglycone in SPI/SG20, SPI/SG40 and SPI/SG80 (total of unconjugated and conjugated forms) was 18, 27 and 46 mg per 200 mL, respectively. For SPI/SG20, 748 g of SPI159 and 52 g of SG were reconstituted in 20 L of filtered water tempered to 40°C, followed by heating with stirring at 70 °C for 30 min during which 80 mL of vanilla flavour was added. The soymilk was then dispensed into four bottles in 5 L quantities and autoclaved at 121°C for 15 min. Two bottles of sterile soymilk (10 L) were cooled to approximately 75 ± 5°C and 200 mL of non-fermented product was dispensed into 45 sterile 200 mL HDPE bottles (Cospak) under aseptic conditions using a 100 mL Trubor® bottle top dispenser (U-Lab). One-hundred millilitre aliquots from three randomly selected bottles of non-fermented soymilk were obtained under aseptic conditions and freeze dried as described in section 3.2.5 for the extraction of isoflavone and analysis using HPLC. Fourteen bottles of non-fermented soymilk were packaged and stored at 4°C prior

to distribution. The remaining 10 L of sterile soymilk was cooled to 40°C prior to inoculation with active culture of BB12. SPI/SG40 and SPI/SG80 were also manufactured in the same manner using 638 g of SPI159 to 162 g of SG and 416 g of SPI159 to 384 g of SG, respectively, per 20 L of filtered water.

7.2.6 Bacterial growth media

Rehydrated MRS agar (de Mann *et al.* 1960) containing additional 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine-HCl was prepared as described in section 3.2.2. For the activation of BB12, 24 g of SPI159 and 6 g of food-grade glucose powder (Prahran Health Foods) per 600 mL of filtered drinking water was reconstituted as described in section 3.2.3. Six hundred millilitres was manufactured and dispensed into glass bottles in 20, 50 and 500 mL quantities and sterilised by autoclaving at 121°C for 15 min.

7.2.7 Fermentation of soymilk by bifidobacteria

BB12 was activated by 3 successive transfers. An inoculum level of 5% (v/v) was used for strain activation and incubation was at 37°C for 20 h. Ten litres of sterile soymilk (SPI/SG20, SPI/SG40 and SPI/SG80) was inoculated with active culture of BB12 (5% v/v). Two hundred millilitres of inoculated soymilk was dispensed into 48 sterile 200 mL HDPE bottles under aseptic conditions using a 100 mL Trubor® bottle top dispenser and incubated at 37°C for 24 h. Three bottles were randomly taken from the batch at 0 and 24 h of incubation and a sample of 100 mL was withdrawn aseptically from each bottle for enumeration of viable BB12 populations (as described in section 3.2.6) and the remainder stored at –20°C. Frozen samples were freeze-dried as described in section 3.2.5 for the extraction of isoflavone and analysis using HPLC. The remaining 42 bottles of fermented soymilk were equally packaged into three cartons and stored at 4°C prior to distribution.

7.2.8 Isoflavone standards

Aglycone standards of genistein, daidzein and glycitein (synthetic) used for both soymilk and urinary isoflavone analyses were purchased from Sigma. β-Glucoside isomers of genistin, daidzin and glycitin were purchased from Indofine Chemical Co. Internal standards of flavone and benzophenone, used for the quantification of isoflavones in soymilk and urine, respectively, were obtained from Sigma. Mixed and single isoflavone standards used for the analysis of isoflavones in soymilk were prepared as described in section 3.2.8.

7.2.9 Reversed-phase HPLC apparatus and reagents

Chromatographic analyses were carried out on a Varian® 9000 series high performance liquid chromatograph (Varian Pty. Ltd., Mulgrave, Victoria, Australia) with auto-sampler (9100), solvent delivery system (9010), polychrom photodiode array UV/VIS detector (9065), and thermostatically controlled column compartment (Alltech Associates Pty. Ltd., Box Hill, Victoria, Australia). An Exsil™ (SGE International Pty. Ltd., Ringwood, Victoria, Australia) C18-ODS (250 x 4.6 mm internal diameter; 5 μm) reversed-phase column was used to separate isoflavone isomers. HPLC grade methanol and acetonitrile were purchased from Merck, and trifluoro-acetic acid, glacial acetic acid, sodium acetate and ammonium acetate from Sigma. All reagents used for soymilk and urinary isoflavone extraction and HPLC analyses were filtered through a 0.5 μm membrane (Millipore®) and mobile phases were degassed using nitrogen.

7.2.10 Extraction and HPLC analysis of isoflavones in soymilk

The extraction of isoflavone isomers (malonyl-, acetyl-, β-glucoside and aglycone forms) from 1.50 g of freeze-dried SPI/SG20, 1.00 g of freeze-dried SPI/SG40 or 0.50 g of freeze-dried SPI/SG80 was performed in duplicate using the method described by Setchell *et al.* (2001). HPLC gradient elution was composed of acetonitrile (solvent A) and 10 mM ammonium acetate buffer containing 0.1% trifluoro-acetic acid (solvent B), set at a flow rate of 1 mL per min (Setchell *et al.* 2001). After 20 μL injection of

sample or isoflavone standard onto the column (25°C), solvent B was set at 100% for 2 min, reduced to 40% over 22 min, followed by 40% for 5 min, increased to 100% over 6 min, and finally 100% for 5 min prior to the next injection. The photodiode array UV/VIS detector was set at 259 nm and the retention times (min) of isoflavone isomers were as follows: malonyldaidzin, 13.7; malonylglycitin, 14.0; malonylgenistin, 14.3; daidzin, 14.6; glycitin, 14.8; genistin, 16.2; acetyldaidzin, 16.9; acetylglycitin, 17.1; acetylgenistin, 18.7; daidzein, 19.2; glycitein, 19.5; genistein, 21.7; and flavone (ISTD), 28.2. Retention times for aglycone and β-glucoside isoflavone isomers were determined using single standards and those of malonyl- and acetyl-glucoside isomers were based on the retention times reported by Setchell *et al.* (2001) under similar HPLC conditions. Quantification of isoflavone isomers in soymilk using multi-level calibration and an ISTD was like that described in section 3.2.10. Isoflavone concentrations were calculated back to wet basis and expressed as μmol per 200 mL of soymilk. Intra- and inter-assay %COV for the concentration of each isoflavone isomer in SG powder (0.50 g) was evaluated like that described in section 3.2.11. Values of intra- and inter-assay %COV for individual isoflavone isomer concentrations in SG powder were equivalent to those reported in section 6.2.7. The intra- and inter-assay %COV for the total isoflavone concentration found in SG powder was ≤ 5 and ≤ 10, respectively, in line with those values reported in section 3.2.11 and 6.2.7.

7.2.11 Analysis of isoflavones in urine specimens

Aliquots from 24-hour pooled urine specimens (section 7.2.3) were analysed for their daidzein, genistein and glycitein concentration. Isoflavones were extracted from urine specimens and analysed in batches over consecutive days with all the specimens from one subject run in the same batch. Extraction of urinary isoflavones was performed as per Franke & Custer (1994) with modifications as follows. Frozen urine aliquots were equilibrated to room temperature, vortex-mixed and centrifuged (Sorvall® RT7 refrigerated centrifuge) at 2700 x g for 10 min. A volume of 20.2 mL of clear supernatant was mixed with 5.0 mL of 0.2 M acetate buffer (pH 4) and 120 μL of benzophenone (10 mg per 50 mL of methanol) and filtered through a C18 solid-phase extraction (SPE) column (Alltech)

preconditioned with 5.0 mL of methanol and 5.0 mL of 0.2 M acetate buffer (pH 4). After passing the entire urine specimen through the SPE column, the SPE column was washed with 2.0 mL of 0.2 M acetate buffer (pH 4) and the isoflavones were eluted with 100% methanol to give exactly 2.0 mL. One hundred microlitres of this eluate was used for the analysis of unconjugated forms of daidzein, genistein and glycitein (50 μL injected onto the column). The residual 1.9 mL of eluate was dried under a stream of nitrogen using a Pierce® nine-needle evaporating unit (Pierce Biotechnology) and then resuspended in 900 μL of freshly prepared mixture comprising 10 mL of 0.2 M acetate buffer (pH 4), 150 mg of ascorbic acid and 500 μL of glucuronidase/sulfatase (sterile filtered crude solution isolated from *Helix Pomatia* type HP-2S) (Sigma). The hydrolysed sample (total of unconjugated and conjugated isoflavones) was then mixed with 1.0 mL of 100% methanol, centrifuged at 4000 x g for 5 min, and 50 μL injected onto the column (25°C). HPLC conditions were as per Xu *et al.* (1994), with the photodiode array UV/VIS detector set at a wavelength of 259 nm. Six mixed standards containing equal concentrations of daidzein, genistein and glycitein (50, 100, 150, 250, 350 and 450 ng per 50 μL) were used for the quantification of isoflavones. Benzophenone (ISTD) was added to each isoflavone standard at a concentration of 600 ng per 50 μL. Single standards were also prepared for peak identification and isoflavone isomer retention times are shown in Figure 7.1. Standards of daidzein and genistein were prepared in methanol and glycitein was prepared in ethanol, as they varied in solubility characteristics. Mixed and single isoflavone standards were dried under a stream of nitrogen using a nine-needle evaporating unit (Pierce Biotechnology) and then resuspended in 100% methanol prior to injection onto the column. Isoflavone concentrations were calculated with respect to the ISTD and multiplied to represent the entire pooled urine specimen (Appendix F).

7.2.12 Statistical analysis

Non-fermented and fermented SPI/SG20, SPI/SG40 and SPI/SG80 were manufactured on three occasions, with three bottles of soymilk randomly selected from each batch and their isoflavone content and viable BB12 populations analysed in duplicate. Hence, populations of BB12 in fermented soymilks

consumed during each supplementation period are reported as a mean ± standard deviation of six replicates, and concentrations of each isoflavone isomer in non-fermented and fermented soymilks consumed during the entire study are presented as a mean ± standard deviation of eighteen replicates. For the FS and NFS group, concentrations of daidzein, genistein and glycitein in 24-hour pooled urine specimens were analysed in duplicate and are presented as a mean ± standard deviation of sixteen replicates. To find significant differences in bodyweight, BMI, nutrient intake, viability of BB12 and isoflavone levels in soymilk, and urinary isoflavone excretion, means were analysed with ANOVA and 95% confidence intervals using Microsoft® Excel Stat Pro™ as described by Albright *et al.* (1999). ANOVA data with a $P<0.05$ was classified as statistically significant (two-sided test).

7.3 RESULTS AND DISCUSSION

7.3.1 BMI and bodyweight

The mean (± standard deviation) BMI (bodyweight) of the NFS and FS group (n = 8) at baseline (that is, a day before the first supplementation period) was 29.3 ± 6.2 kg/m^2 (71.3 ± 13.4 kg) and 24.6 ± 5.4 kg/m^2 (61.9 ± 11.3 kg), respectively, with no significant difference in either BMI ($P=0.13$) or bodyweight ($P=0.15$) between the two groups. No significant fluctuations in bodyweight were observed during the entire span of supplementation and washout periods for the NFS and FS group ($P>0.05$). Hence, the mean BMI of the NFS and FS group after the final supplementation period was 28.9 ± 5.6 and 24.3 ± 5.3 kg/m^2, respectively, similar to their respective baseline values ($P>0.05$).

7.3.2 Intake of dietary nutrients, viable bifidobacteria and isoflavones

The mean (± standard deviation) daily energy intake for women in the FS and NFS group ranged between 5698.8 ± 1478.9 and 6445.9 ± 1568.6 KJ per day, with no significant differences in daily energy intake between the two groups during supplementation periods ($P=0.9$). Mean daily intake of macronutrients for the FS and NFS group during supplementation are shown in Figure 7.2. Both groups

of women consumed an average of 20% of energy in the form of protein, 44% of energy as carbohydrates and 36% as fat (total intake of polyunsaturated, monounsaturated and saturated fats) per day during supplementation. There were no significant differences in protein (P=0.8), carbohydrate (P=0.7), fat (P=0.9) and dietary fibre (P=0.9) intake (g per day) between the FS and NFS group during supplementation periods.

According to the protein content of SPI159 and SG (section 6.2.1), non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 comprised 6.96, 6.40 and 5.28 g of protein per 200 mL serving, respectively. The concentration of carbohydrate (and fibre) in non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 was 0.42 (0.01 g), 0.66 (0.05 g) and 1.13 (0.12 g) g per 200 mL serving, respectively. The total concentration of polyunsaturated, monounsaturated and saturated fats in non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 was 0.38, 0.51 and 0.78 g per 200 mL serving, respectively. Each of the soymilks contributed relatively little to the mean intake of macronutrients ingested per day by women in the FS and NFS group, at approximately 8.5%, 0.4%, 1.0% and 0.3% of the daily intake of protein, carbohydrate, fat and fibre, respectively. Fermented SPI/SG20, SPI/SG40 and SPI/SG80 may have contained a slightly lower level of protein, carbohydrate, fat and fibre than their non-fermented counterparts, as each of these nutrients could have been metabolised as a growth substrate during fermentation of bifidobacteria (Kamaly, 1997; Hou *et al.* 2000).

Viable populations of BB12 in fermented SPI/SG20, SPI/SG40 and SPI/SG80 (200 mL serving) consumed by women in the FS group during supplementation ranged from 7.60 ± 0.06 to 8.87 ± 0.08 $\log_{10}$ CFU per mL. This level of viable bifidobacteria exceeded the minimum level considered to be of therapeutic dosage at 6 $\log_{10}$ CFU per mL (Gomes & Malcata, 1999). Such high numbers of bifidobacteria compensate for the possible reduction in viable cells during passage through the stomach and intestine. There were no significant differences between BB12 populations found in fermented

SPI/SG20, SPI/SG40 and SPI/SG80 (P=0.07). Furthermore, similar populations of BB12 were ingested during each supplementation period (P=0.08).

Mean concentrations of isoflavone isomers in non-fermented and fermented SPI/SG20, SPI/SG40 and SPI/SG80 ingested per day (via 200 mL serving) during soymilk supplementation are shown in Table 7.1; each value is expressed as μmol so as to be compared to the levels of isoflavone (μmol per day) excreted in urine. Non-fermented SPI/SG20, SPI/SG40 and SPI/SG80, which were formulated to contain 20, 40 and 80 mg isoflavone per 200 mL, were equivalent to 64.11, 101.80 and 172.28 μmol aglycone constituents per 200 mL, respectively. The highest daily dose of isoflavone via SPI/SG80 was roughly 1.7 and 2.6 times the isoflavone concentration of SPI/SG40 and SPI/SG20, respectively (P<0.001). Non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 comprised 10%, 9% and 7% of its isoflavone level as unconjugated aglycone, respectively. Fermentation of SPI/SG20, SPI/SG40 and SPI/SG80 by BB12 significantly increased the proportion of aglycone structures to 69%, 57% and 36% of total isoflavone, respectively (P<0.001). Fermented SPI/SG40 and SPI/SG80 contained the highest concentration of aglycone structures at 57.74 and 61.67 μmol per 200 mL, respectively; significantly higher than the other soymilks (P<0.001). However, changes in isoflavone isomer composition were less apparent in SPI/SG80 after fermentation. In Chapter 6.0, it was reported that the β-glucosidase levels produced by BB12 during soymilk fermentation were insufficient to hydrolyse high levels of isoflavone glucoside, in the range found in SPI/SG80. Furthermore, SPI/SG80 comprised the greatest concentration of glycitin, malonylglycitin and acetylglycitin (approximately 35% of total isoflavone), which appear to be poorly hydrolysed by β-glucosidase during soymilk fermentation by BB12 (Chapter 6.0). The high concentration of glycitein and its glucosides in SPI/SG80 was due to the greater proportion of SG powder in the soymilk formulation.

7.3.3 Urinary isoflavone excretion after consumption of fermented and non-fermented soymilk

Inter-individual variation in intestinal microflora has shown to affect the bioavailability of isoflavones (Hendrich, 2002). The influence of diet on the bioavailability of isoflavones has also been observed (Setchell *et al.* 1984; Kelly *et al.* 1993; Lampe *et al.* 1998). The ingestion of viable bifidobacteria to modulate intestinal microflora and its possible effect on the bioavailability of isoflavones has not been studied. Changes in intestinal microflora occur from birth to old age; with young adult women having a different intestinal microbial ecology from those women in their postmenopausal years (Ballongue, 1993). Mitsuoka (1984) reported that numbers of *Bifidobacterium* in adult stools, particularly those of the elderly, were significantly lower than the populations of *Bifidobacterium* found in the stool samples of children. Therefore, due to the relevance of isoflavone-rich soybean foods to postmenopausal women and considering the variation in intestinal microflora over different stages of life, we limited the selection of subjects to women between the ages of 45 to 65 years. Furthermore, we segregated those women consuming fermented soymilk from those consuming the non-fermented product, as we expected changes in intestinal microbial composition in the group of women consuming bifidobacteria.

Mean concentrations of isoflavone excreted in urine by the FS and NFS group at 4, 13 and 14 days of soymilk supplementation are shown in Table 7.2, with the concentrations representing the sum of unconjugated forms of daidzein, genistein and glycitein and their deconjugated glucuronide and sulfate isomers. The proportion of unconjugated isoflavone metabolites excreted in urine by the FS and NFS group ranged between 0.6% and 3.0% of total urinary isoflavone. Similarly, Zhang *et al.* (2003) found that the percentage of aglycone forms excreted in urine by young adult women after the ingestion of non-fermented soymilk was 4 to 5% of total isomers, with daidzein and genistein glucuronides representing 73% and 71% of total daidzein and genistein excreted in urine, respectively. As shown in Table 7.2, women from both the FS and NFS group excreted the greatest level of isoflavone (38 to 53 μmol per day) during supplementation of SPI/SG80, which contained the highest dose of isoflavone (Table 7.1). The level of isoflavone excreted in urine during supplementation of SPI/SG80 was

significantly greater than the levels excreted during supplementation of SPI/SG20 and SPI/SG40 for both the FS and NFS group on day 4 ($P<0.001$), 13 ($P<0.001$) and 14 ($P<0.001$) (Table 7.2). Hence, a higher ingested dose of isoflavone via SPI/SG80 appeared to enhance the amount of isoflavone absorbed and subsequently excreted during soymilk supplementation. Likewise, Xu *et al.* (1994) reported that total urinary isoflavones increased significantly with increasing dose ($P<0.05$).

Ingestion of fermented soymilk did not appear to enhance the urinary excretion of isoflavone at any of the isoflavone dosages (SPI/SG20, SPI/SG40 and SPI/SG80), as there was no significant difference in urinary isoflavone concentrations between the FS and NFS group after 4, 13 and 14 days ($P>0.05$) (Table 7.2). Even though fermented SPI/SG20, SPI/SG40 and SPI/SG80 contained a significantly greater proportion of isoflavone in an aglycone configuration than its non-fermented counterpart, urinary isoflavone recovery was also similar for both the FS and NFS group ($P>0.05$). Mean urinary isoflavone recovery during supplementation of fermented (and non-fermented) SPI/SG20, SPI/SG40 and SPI/SG80 was approximately 34% (39%), 30% (24%) and 28% (31%) of the ingested isoflavone dose, respectively. In comparison, Hendrich *et al.* (2001) investigated the recovery of isoflavones in urine, plasma and faecal specimens collected from two groups of women consuming either aglycone-rich or glucoside-rich SG concentrate and found that the bioavailability of isoflavone was similar after 4 and 7 days of supplementation. Furthermore, after a single serving of an isoflavone aglycone-rich SG beverage, Richelle *et al.* (2002) found that the plasma pharmacokinetics of isoflavones ($T½_{abs}$, $T½_{elim}$, C_{max} and $AUC_{0\text{-}34h}$) in postmenopausal women were similar when the same women (in a crossover design) ingested a glucoside-rich SG equivalent ($P>0.05$). In contrast, Setchell *et al.* (2001) reported that isoflavone glucosides actually showed greater bioavailability than aglycones in women ingesting a single dose, according to the AUC of the plasma appearance and disappearance concentrations, and this appeared to be due to the hydrolytic actions of intestinal β-glucosidases. To date, there is no evidence to indicate that ingesting isoflavones in an aglycone-rich configuration enhances their bioavailability after either a single dose or consistent daily consumption.

Following the collection of a urine specimen at day 0, we allowed three days of soymilk intake for women to reach a steady state of urinary isoflavone excretion and compared this to two 24-hour pooled urine specimen collections at the completion of supplementation. There were no significant differences between levels of total urinary isoflavone and individual isomers (daidzein, genistein and glycitein) excreted on day 4, 13 and 14 of supplementation of fermented and non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 ($P>0.05$). Hence, a steady level of isoflavone absorption and urinary excretion appeared to have been achieved at each isoflavone dose for both fermented and non-fermented soymilk. Setchell *et al.* (2003) stated that optimum steady-state serum isoflavone concentrations would be expected from modest intakes of soy foods consumed regularly throughout the day rather than from a single highly enriched product. Each subject in our study was required to consume the daily 200 mL serving of soymilk as two portions, before breakfast and before dinner.

The positive correlation between mean urinary excretion of total isoflavone per day versus the mean amount of total isoflavone ingested per day during supplementation of non-fermented and fermented soymilk is shown in Figure 7.3A and 7.3B, respectively. According to the computed lines of regression, there was a clearer linear relationship between isoflavone excretion and dose amongst fermented soymilks ($R^2=0.9993$) (Figure 7.3B) compared to the linearity observed for non-fermented soymilks containing equivalent dosages of isoflavone ($R^2=0.8865$) (Figure 7.3A). Even though the consumption of fermented soymilk did not enhance urinary isoflavone excretion ($P>0.05$) (Table 7.2), it did possibly reduce inter-individual variability in isoflavone absorption and urinary excretion indicated by the high linearity in dose response (Figure 7.3B). This is reflected in Table 7.2, with values of standard deviation for urinary isoflavone excretion generally higher amongst the NFS group at each isoflavone dose.

The distinct linear dose-response for the FS group (Figure 7.3B) may have been due to the higher proportion of aglycone structures ingested via the fermented soymilks or the consumption of viable BB12. Turner *et al.* (2003) stated that the composition of intestinal microflora appears to play a key

role in the intestinal biotransformation and absorption of isoflavones and may cause significant inter-individual variability. Those women consuming the fermented soymilk may have established a more consistent intestinal microflora during supplementation by increasing the populations of β-glucosidase-producing bifidobacteria and adjusting intestinal microbial balance in favour of other saccharolytic enzyme-producing lactic acid bacteria. Hence, greater variation in gut microflora between women consuming the non-fermented soymilks may have caused the poorer linearity in dose-response (Figure 7.3A). Furthermore, non-fermented soymilks most likely contained a greater concentration of oligosaccharide compared to fermented soymilk; oligosaccharides are metabolised by BB12 during fermentation (Chapter 5.0). Soybean oligosaccharides are classified as prebiotics, which stimulate the growth of bifidobacteria and other lactic acid producing bacteria in the intestinal tract (Masai *et al.* 1987). This may have enhanced intestinal microbial metabolic activity in some subjects in the NFS group, potentially influencing the extent of isoflavone biotransformation and absorption. Not to be discounted is the deglycosylation of isoflavones by mammalian intestinal glucosidases (Day *et al.* 1998; Setchell *et al.* 2001) and the extent of their effect on inter-individual variation in isoflavone absorption within the FS and NFS group. Furthermore, Rowland *et al.* (2000) reported that dietary intake of carbohydrate and fat also influenced the intestinal biotransformation of isoflavones. However, since both the FS and NFS group had a similar daily intake of carbohydrate (P=0.7) and fat (P=0.9), we believe dietary intake had a lesser influence on inter-individual variation in urinary isoflavone excretion within each group and the difference in linearity in isoflavone dose response shown in Figure 7.3.

7.3.4 Bioavailability of daidzein, genistein and glycitein from fermented and non-fermented soymilk

The mean concentrations of daidzein, genistein and glycitein (total of conjugated and unconjugated forms) excreted in urine by the FS and NFS group at 4, 13 and 14 days of soymilk supplementation are shown in Table 7.3, and the percentage of ingested dose of daidzein, genistein and glycitein recovered in urine shown in Figure 7.4. On days 4, 13 and 14, the urinary excretion of daidzein was significantly

greater than glycitein and genistein during supplementation of both fermented and non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 ($P<0.001$) (Table 7.3). Similarly, Richelle *et al.* (2002) reported that after the ingestion of an SG beverage, postmenopausal women excreted greater concentrations of daidzein in urine followed by glycitein and genistein, with urinary recovery of daidzein at approximately 53% compared to 34% and 19% for glycitein and genistein, respectively. Furthermore, Zhang *et al.* (2001) fed men and women (between the ages of 19 to 35 years) SG and found that urinary recovery of daidzein was higher than glycitein ($P>0.05$), but the recovery of daidzein and glycitein was significantly greater than genistein ($P<0.05$). Figure 7.4 shows that the percentage recovery of daidzein in urine was significantly greater than both glycitein and genistein during supplementation of both fermented and non-fermented soymilk at all three isoflavone dosages ($P<0.001$). Percentage recovery of glycitein in urine was also greater than genistein during supplementation of fermented and non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 ($P>0.05$) (Figure 7.4). Considering the profound differences in gut microflora between subjects from different countries and cultural backgrounds as well as from different age groups (Mitsuoka, 1984), there appeared to be a consistent pattern in the urinary excretion and percentage recovery of isoflavone isomers found in our study and in those studies by Zhang *et al.* (2001) and Richelle *et al.* (2002). That is, the greatest of proportion of isoflavone in urine was found as daidzein, the smallest proportion as genistein, and the proportion of glycitein found somewhere in between.

According to urinary isoflavone profiles shown in Table 7.3 and Figure 7.4, genistein appears to be less bioavailable than daidzein and glycitein. This is consistent with previous studies by Xu *et al.* (1994, 1995) and Lu *et al.* (1996), reporting substantially higher urinary recovery of daidzein compared with genistein. Pharmacokinetic studies by King & Bursill (1998) also found the urinary recovery of daidzein (62%) to be significantly greater than genistein (22%) ($P<0.001$). However, King & Bursill (1998) concluded that the bioavailability of daidzein and genistein were similar considering that the ratio of the AUC for genistein and daidzein was equal to ratio of the concentration of these respective

isoflavones in the soy meal ingested. Setchell *et al.* (2001) administered a 50 mg-dose of daidzein, daidzin, genistein or genistin to four groups of premenopausal women and found that the systemic bioavailability of genistein [mean AUC of 4.54 μg/(mL·h)] was much greater than daidzein [mean AUC of 2.94 μg/(mL·h)]. Nevertheless, the results from these earlier urinary and plasma pharmacokinetic studies only involved the administration of a single dose. Over a period of daily soy isoflavone ingestion the bioavailability of daidzein, genistein and glycitein may begin to differ depending on the response of intestinal microflora to the presence of these compounds. Hence, the lower concentrations of genistein in urine may be due to its greater susceptibility to microbial breakdown in the intestinal tract and therefore being less bioavailable after chronic soy ingestion. Xu *et al.* (1995) reported that genistein was more susceptible than daidzein to biotransformation by intestinal bacteria during anaerobic incubations of human faecal samples.

Women consuming both the fermented and non-fermented SPI/SG80 excreted significantly greater levels of daidzein (ranging from 28 to 37 μmol per day) on days 4, 13 and 14 of supplementation, compared with the consumption of SPI/SG20 and SPI/SG40 (Table 7.3). The greater level of daidzein and its glucosidic conjugates in SPI/SG80 (Table 7.1) appeared to account for the increased absolute absorption and urinary excretion of this isomer in the FS and NFS group. However, the percentage recovery of daidzein in urine tended to be greater for women consuming fermented and non-fermented SPI/SG20 (57% and 63% of dose, respectively) (P>0.05) (Figure 7.4). Concentrations of daidzein and its glucosides at approximately 22.6 μmol (5.7 mg) aglycone constituents per 200 mL (found in SPI/SG20) appeared to be less susceptible to microbial breakdown (reductive metabolism) in the gastrointestinal tract and more bioavailable according to percentage recovery of dose. Lower doses of daidzein may be absorbed more effectively in the proximal gastrointestinal tract, reflecting more complete absorption. With higher doses of daidzein, the fraction not absorbed proximally may be absorbed distally in the gastrointestinal tract, where there are greater populations of intestinal bacteria possibly biotransforming daidzein into equol, dihydrodaidzein and other metabolites prior to

absorption, accounting for the lower percentage recovery of dose for SPI/SG40 and SPI/SG80 (Figure 7.4). Limited or saturable absorption of daidzein in the proximal gastrointestinal tract may be the reason for higher ingested doses of daidzein to pass into the ileum and colon. This may be influenced by the efficacy of mammalian glucosidases present in the proximal intestinal tract to hydrolyse glucosidic isomers of daidzein into a bioavailable aglycone configuration, as there are considerably lower populations of bacteria in this region compared to the ileum and colon (Turner *et al.* 2003).

In contrast to the urinary excretion of daidzein, Table 7.3 shows that there were no significant differences in the urinary excretion of genistein during supplementation of fermented and non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 at day 4, 13 and 14 ($P>0.05$). In this case, increasing the dose of genistein was not associated with increased urinary excretion of this isomer, possibly due to its greater susceptibility to intestinal microbial breakdown in comparison to daidzein (Xu *et al.* 1995) or could reflect a limited or saturable absorptive capacity in the gastrointestinal tract. However, it can be seen in Table 7.1 that the difference in dosage levels of genistein and its glucosides from each of the fermented and non-fermented forms of SPI/SG20, SPI/SG40 and SPI/SG80 were not as distinct as those of daidzein between these soymilks. Furthermore, comparable genistein dosage levels between soymilks may have caused the similar percentage recovery of genistein in urine between fermented and non-fermented SPI/SG20, SPI/SG40 and SPI/SG80, in the range of 14 to 17% of ingested dose ($P>0.05$) (Figure 7.4). In comparison, Hutchins *et al.* (1995) reported that the mean urinary recovery of genistein amongst a group of men consuming fermented soybean pieces (tempeh) was only 1.9% at a dosage of 44.5 μmol genistein per day (unconjugated and glucosidic isomers), equivalent to the dosage of fermented SPI/SG80 (44.4 μmol genistein per day). The mean urinary recovery of unconjugated and glucosidic isomers of daidzein from the same group of men consuming tempeh was 9.7% at a dosage of 21.3 μmol daidzein per day (Hutchins *et al.* 1995), considerably lower than the mean urinary recovery of daidzein amongst the FS group (57% of ingested dose) ingesting an equivalent dosage of daidzein via SPI/SG20 (23.3 μmol daidzein per day). The greater percentage recovery of daidzein and genistein

in our study may have been due to the greater proportion of unconjugated daidzein in fermented SPI/SG20 (73% of dose) and unconjugated genistein in fermented SPI/SG80 (53% of dose) compared to 22% unconjugated daidzein and 10% unconjugated genistein found in tempeh reported by Hutchins *et al.* (1995). Alternatively, soymilk has a greater proportion of simple isoflavone glucosides (β-glucoside isomers) than do less processed soy foods, possibly easier to hydrolyse into bioavailable aglycones via mammalian and microbial glucosidases (King & Bursill, 1998).

Even though the urinary excretion of glycitein resembled a linear response to dose in the FS group, there were no significant differences at day 4, 13 or 14 to suggest that increasing the dosage level of glycitein significantly enhanced the absorption and urinary excretion of this isomer (Table 7.3). Like the urinary recovery of daidzein, significantly greater percentage recovery (in the range of 30 to 35%) was observed when ingesting the lowest level of glycitein via fermented and non-fermented SPI/SG20 ($P<0.001$) (Figure 7.4), comprising a glycitein concentration of approximately 12.0 μmol (3.4 mg) per 200 mL serving. Figure 7.4 also shows that women consuming fermented SPI/SG40 tended to have a greater percentage recovery of glycitein (22% of dose) in comparison to the ingestion of non-fermented SPI/SG40 (urinary recovery of 18%) ($P=0.13$). Similarly, percentage recovery of daidzein in urine also appeared greater amongst women consuming fermented SPI/SG40 (48% of dose) compared to women consuming non-fermented SPI/SG40 (urinary recovery of 41%) ($P=0.14$). However, there were no significant differences in the urinary percentage recovery of daidzein and glycitein found between the FS and NFS group ($P>0.05$) (Figure 7.4) to support the possible enhanced bioavailability of these isomers from fermented soymilk.

In an earlier crossover study, Hutchins *et al.* (1995) assigned an adequate 12-day washout period between each of their 9-day feeding periods. Since our study involved daily soymilk ingestion for up to 14 days, we decided to implement a similar time period to our washout phase as used by Hutchins *et al.* (1995). Previous pharmacokinetic studies involving a single serving of soy food generally used shorter

washout periods of 5 to 6 days (King & Bursill, 1998; Richelle *et al.* 2002), which we believed may not have been adequate considering the longer exposure to isoflavones and the potential for enterohepatic recirculation. Nevertheless, urinary isoflavones were detected on day 0 (baseline) of each of the isoflavone dosages ingested (Table 7.2), suggesting that the 14-day washout period implemented in the crossover design was not long enough to fully excrete circulating isoflavone metabolites in each of the women. Alternatively, isoflavones detected in baseline urine specimens may have been the result of women unknowingly ingesting foods or ingredients containing isoflavones, which were supposed to be excluded during supplementation and washout periods. This may have been clarified if each subject recorded their dietary intake during the washout periods so as to assess whether the presence of isoflavones at baseline were due to physiological or dietary reasons. However, considering the widespread use of soy ingredients in Western foods (e.g. bread and cereal products) and the presence of isoflavone precursors in dairy milk (King *et al.* 1998), it is likely that a large proportion of the Western population excrete detectable amounts of isoflavone in urine even if soy foods are not a normal component of the Western diet (Kelly *et al.* 1993). Of the individual isomers, low concentrations of both daidzein and genistein were detected at baseline for both the FS and NFS group (Table 7.3). In contrast, glycitein was not detected in baseline urine specimens (Table 7.3), with 14 days appearing to be an adequate period to wash out glycitein from the circulatory system. Then again, if dietary intake was the reason for the presence of isoflavones in baseline urine specimens, glycitein may not have been detected because it is found at considerably lower levels than daidzein and genistein in conventional soy foods and ingredients found in Australia (King & Bignell, 2000).

From a health perspective, isoflavones may be of greater benefit to those women who are able to retain biologically active isoflavone metabolites in their circulatory system for longer periods of time after discontinuing the intake of soy foods. In the NFS group, the woman who excreted the greatest level of isoflavone after two 14-day washout periods also had the highest BMI, at approximately 39 kg/m^2.

Isoflavones in an aglycone form are lipid soluble; hence, women with a higher percentage of body fat may have a greater tendency to retain isoflavones.

7.4 CONCLUSIONS

Fermentation of each soymilk with BB12 enriched the level of isoflavone aglycone. Additionally, each soymilk was able to support the viability of BB12 during refrigerated storage at levels able to effectively modulate intestinal microbial balance. However, there was no strong evidence to suggest that fermenting soymilk with bifidobacteria improved the bioavailability of isoflavone in postmenopausal women over 14 days of daily soymilk ingestion. Levels of total isoflavone (unconjugated and conjugated forms) excreted in urine and urinary isoflavone recovery was similar for both groups of women consuming either fermented or non-fermented soymilks. Nevertheless, ingestion of fermented soymilk appeared to reduce inter-individual variation in isoflavone absorption. Of the individual isomers, percentage recovery of daidzein and glycitein in urine tended to be greater amongst women consuming fermented soymilk at a daily dosage of 40 mg isoflavone. Increasing the dosage of isoflavone correlated positively with urinary excretion of isoflavone, but urinary recovery of isoflavone was inversely related to dosage level. Hence, a modest isoflavone dosage ranging from 20 to 30 mg aglycone constituents per day may provide the most bioavailable source of isoflavone.

Table 7.1 Concentration of isoflavone isomers (μmol per 200 mL serving) in non-fermented and fermented SPI/SG20, SPI/SG40 and SPI/SG80 (mean ± standard deviation; n = 18)

Isoflavone	Non-Fermented			Fermented[1]		
Isomer	SPI/SG20	SPI/SG40	SPI/SG80	SPI/SG20	SPI/SG40	SPI/SG80
Daidzein	3.42±0.93^{f}	4.77±0.97^{ef}	6.24±0.98^{de}	17.01±1.08^{c}	23.84±1.96^{b}	29.00±4.87^{a}
Genistein	2.03±0.63^{de}	2.26±0.64^{cde}	1.84±0.19^{e}	27.16±1.97^{a}	28.52±2.13^{a}	23.62±4.10^{b}
Glycitein	0.74±0.32^{f}	2.06±0.76^{e}	3.80±0.64^{cd}	3.01±0.89^{de}	5.38±0.66^{b}	9.05±1.56^{a}
Aglycones[2]	6.19±1.71^{e}	9.09±2.10^{de}	11.88±1.66^{cd}	47.18±3.67^{b}	57.74±4.39^{a}	61.67±10.19^{a}
Daidzin	15.23±0.82^{d}	25.82±1.62^{c}	49.04±2.40^{a}	4.53±0.89^{f}	10.70±1.32^{e}	30.51±6.21^{b}
Genistin	25.31±1.46^{c}	28.40±1.63^{b}	31.13±1.98^{a}	5.47±1.21^{f}	7.45±1.06^{ef}	17.41±4.26^{d}
Glycitin	9.91±0.82^{ef}	23.08±1.39^{c}	50.18±3.09^{a}	7.04±1.11^{f}	17.24±1.71^{d}	41.23±4.90^{b}
β-Glucosides[2]	50.45±2.98^{d}	77.30±4.50^{c}	130.35±7.25^{a}	17.04±3.14^{f}	35.39±3.89^{e}	89.15±15.20^{b}
Malonyldaidzin	0.80±0.86^{cd}	1.54±0.67^{abcd}	2.18±0.93^{a}	0.61±0.82^{d}	1.15±0.66^{bcd}	1.69±1.14^{abc}
Malonylgenistin	0.83±0.36^{cde}	1.69±0.38^{b}	2.84±0.94^{a}	0.31±0.33^{e}	0.64±0.30^{de}	2.26±0.83^{ab}
Malonylglycitin	0.16±0.20^{d}	0.44±0.19^{bcd}	0.86±0.24^{a}	0.60±0.50^{abc}	0.43±0.20^{cd}	0.84±0.30^{a}
Malonylglucosides[2]	1.79±1.28^{de}	3.67±0.88^{bc}	5.88±1.84^{a}	1.52±0.99^{e}	2.22±0.97^{cde}	4.79±2.12^{ab}
Acetyldaidzin	2.39±0.46^{de}	4.87±0.43^{c}	10.30±0.95^{a}	1.11±0.77^{f}	2.05±0.46^{ef}	5.69±1.53^{bc}
Acetylgenistin	1.70±0.58^{cd}	2.88±0.42^{b}	4.77±0.91^{a}	0.40±0.64^{ef}	0.36±0.38^{f}	1.15±1.22^{def}
Acetylglycitin	1.59±0.44^{e}	3.99±0.37^{c}	9.10±0.81^{a}	0.84±0.39^{f}	2.09±0.32^{de}	6.20±1.12^{b}
Acetylglucosides[2]	5.68±1.35^{de}	11.74±1.16^{c}	24.17±2.56^{a}	2.35±1.57^{f}	4.50±1.10^{ef}	13.04±3.61^{bc}
Total isoflavone[3]	64.11±6.55^{e}	101.80±7.29^{bc}	172.28±11.83^{a}	68.09±7.55^{de}	99.85±8.27^{c}	168.65±13.13^{a}

SPI/SG20: Soymilk containing 20 mg isoflavone per 200 mL; SPI/SG40: Soymilk containing 40 mg isoflavone per 200 mL; SPI/SG80: Soymilk containing 80 mg isoflavone per 200 mL.

Means in the same row with different superscript are significantly different ($P<0.05$).

[1]Inoculated with active culture of BB12 (*B. animalis*) and incubated for 24 h at 37 °C.

[2]Mean total of three respective isomers.

[3]Mean total of malonyl-, acetyl-, β-glucoside and aglycone isomers.

Table 7.2 Urinary excretion of total[1] and unconjugated isoflavones (μmol per day[2]) by 2 groups of 8 women[3] during supplementation of fermented and non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 (mean ± standard deviation; n = 16[4])

Day of 24-hour pooled urine collection	Non-Fermented			Fermented		
	SPI/SG20	SPI/SG40	SPI/SG80	SPI/SG20	SPI/SG40	SPI/SG80
Baseline[5]	0.44±0.58^{a}	0.41±0.33^{a}	0.22±0.28^{a}	0.20±0.21^{a}	0.53±0.70^{a}	0.53±0.49^{a}
Day 4						
Total isomers	24.30±7.22^{bcde,A}	20.42±8.33^{e,A}	51.01±11.61^{a,A}	20.67±8.11^{de,A}	23.33±7.85^{cde,A}	38.29±18.14^{a,A}
Unconjugated isomers	0.15±0.05^{a}	0.58±0.85^{a}	0.69±0.63^{a}	0.55±0.53^{a}	0.56±0.42^{a}	0.31±0.22^{a}
Day 13						
Total isomers	23.83±8.46^{de,A}	26.08±9.78^{cde,A}	45.42±17.68^{a,A}	19.91±6.44^{e,A}	31.01±13.43^{bcde,A}	43.27±15.19^{a,A}
Unconjugated isomers	0.13±0.09^{a}	0.77±0.99^{a}	1.23±1.04^{a}	0.36±0.23^{a}	1.03±0.71^{a}	0.64±0.40^{a}
Day 14						
Total isomers	19.91±9.76^{e,A}	22.75±13.19^{cde,A}	52.54±29.87^{a,A}	21.01±5.84^{de,A}	28.24±12.11^{bcde,A}	43.62±17.63^{a,A}
Unconjugated isomers	0.17±0.13^{a}	0.63±0.48^{a}	0.66±0.59^{a}	0.31±0.32^{a}	0.75±0.60^{a}	0.62±0.38^{a}

SPI/SG20: Soymilk containing 20 mg isoflavone per 200 mL; SPI/SG40: Soymilk containing 40 mg isoflavone per 200 mL; SPI/SG80: Soymilk containing 80 mg isoflavone per 200 mL.

Means in the same row and column with different lower- and upper-case superscript, respectively, are significantly different ($P<0.05$).

[1]Mean total of daidzein, genistein and glycitein present after hydrolysis with β-glucuronidase/sulfatase (conjugated and unconjugated forms).

[2]Concentration of isoflavone in 24-hour pooled urine specimen.

[3]Consisting of two separate groups consuming either each of the fermented or non-fermented soymilks.

[4]Eight 24-hour pooled urine specimens (one per subject) analysed in duplicate.

[5]Total concentration of isoflavone isomers in 24-hour pooled urine specimen collected one day before soymilk supplementation.

Table 7.3 Urinary excretion of total[1] daidzein, genistein and glycitein (μmol per day[2]) by 2 groups of 8 women[3] during supplementation of fermented and non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 (mean ± standard deviation; n = 16[4])

Isoflavone isomer	**Non-Fermented**			**Fermented**		
Day of 24-hour urine collection	**SPI/SG20**	**SPI/SG40**	**SPI/SG80**	**SPI/SG20**	**SPI/SG40**	**SPI/SG80**
Daidzein						
Baseline[5]	0.23±0.37^{a}	0.26±0.23^{a}	0.16±0.25^{a}	0.11±0.14^{a}	0.29±0.39^{a}	0.27±0.37^{a}
Day 4	15.00±5.41^{cde,A}	11.44±7.72^{e,A}	35.87±6.35^{a,A}	12.33±6.28^{de,A}	15.09±5.74^{bcde,A}	27.49±15.94^{a,A}
Day 13	13.93±4.60^{de,A}	17.42±5.86^{cde,A}	32.00±10.18^{a,A}	13.69±5.79^{e,A}	20.50±8.93^{bcde,A}	32.00±12.07^{a,A}
Day 14	11.93±5.85^{e,A}	15.01±8.16^{cde,A}	37.07±19.31^{a,A}	13.06±4.04^{de,A}	18.54±7.80^{bcde,A}	32.56±13.23^{a,A}
Genistein						
Baseline[5]	0.17±0.22^{a}	0.13±0.16^{a}	0.04±0.04^{a}	0.05±0.13^{a}	0.19±0.32^{a}	0.09±0.08^{a}
Day 4	5.01±1.60^{a,A}	4.49±3.64^{a,A}	5.02±1.66^{a,A}	6.06±3.45^{a,A}	5.23±2.94^{a,A}	5.35±4.27^{a,A}
Day 13	4.95±2.45^{a,A}	6.68±5.97^{a,A}	5.28±2.63^{a,A}	3.96±1.97^{a,A}	6.07±3.48^{a,A}	5.32±1.88^{a,A}
Day 14	5.26±3.05^{a,A}	4.90±4.00^{a,A}	6.18±4.72^{a,A}	5.94±2.48^{a,A}	5.77±2.82^{a,A}	5.36±3.75^{a,A}
Glycitein						
Baseline[5]	ND	ND	ND	ND	ND	ND
Day 4	4.29±1.55^{d,A}	5.26±3.48^{bcd,A}	10.12±5.51^{a,A}	4.31±1.68^{cd,A}	5.90±4.15^{abcd,A}	7.11±4.21^{abcd,A}
Day13	5.10±4.66^{ab,A}	6.77±5.06^{ab,A}	9.40±6.34^{a,A}	2.62±1.41^{b,B}	5.94±3.11^{ab,A}	5.94±1.93^{ab,A}
Day 14	3.65±2.87^{cd,A}	4.43±3.31^{bcd,A}	10.38±6.49^{a,A}	3.52±1.88^{d,AB}	5.47±3.12^{abcd,A}	7.88±5.08^{abcd,A}

SPI/SG20: Soymilk containing 20 mg isoflavone per 200 mL; SPI/SG40: Soymilk containing 40 mg isoflavone per 200 mL; SPI/SG80: soymilk containing 80 mg isoflavone per 200 mL.

ND: Not detected in 20 mL urine specimen using an injection volume of 50 μL.

Means in the same row (arranged as 6 values across) and column (arranged as three values down) with different lower- and upper-case superscript, respectively, are significantly different ($P<0.05$).

[1]Concentration of daidzein, genistein and glycitein after β-glucuronidase/sulfatase deconjugation (conjugated and unconjugated forms).

[2]Concentration of daidzein, genistein and glycitein in 24-hour pooled urine specimen.

[3]Consisting of two segregated groups consuming either each of the fermented or non-fermented soymilks.

[4]Eight 24-hour pooled urine specimens (one per subject) analysed in duplicate.

[5]Concentration of daidzein, genistein and glycitein in 24-hour pooled urine specimen collected one day before soymilk supplementation.

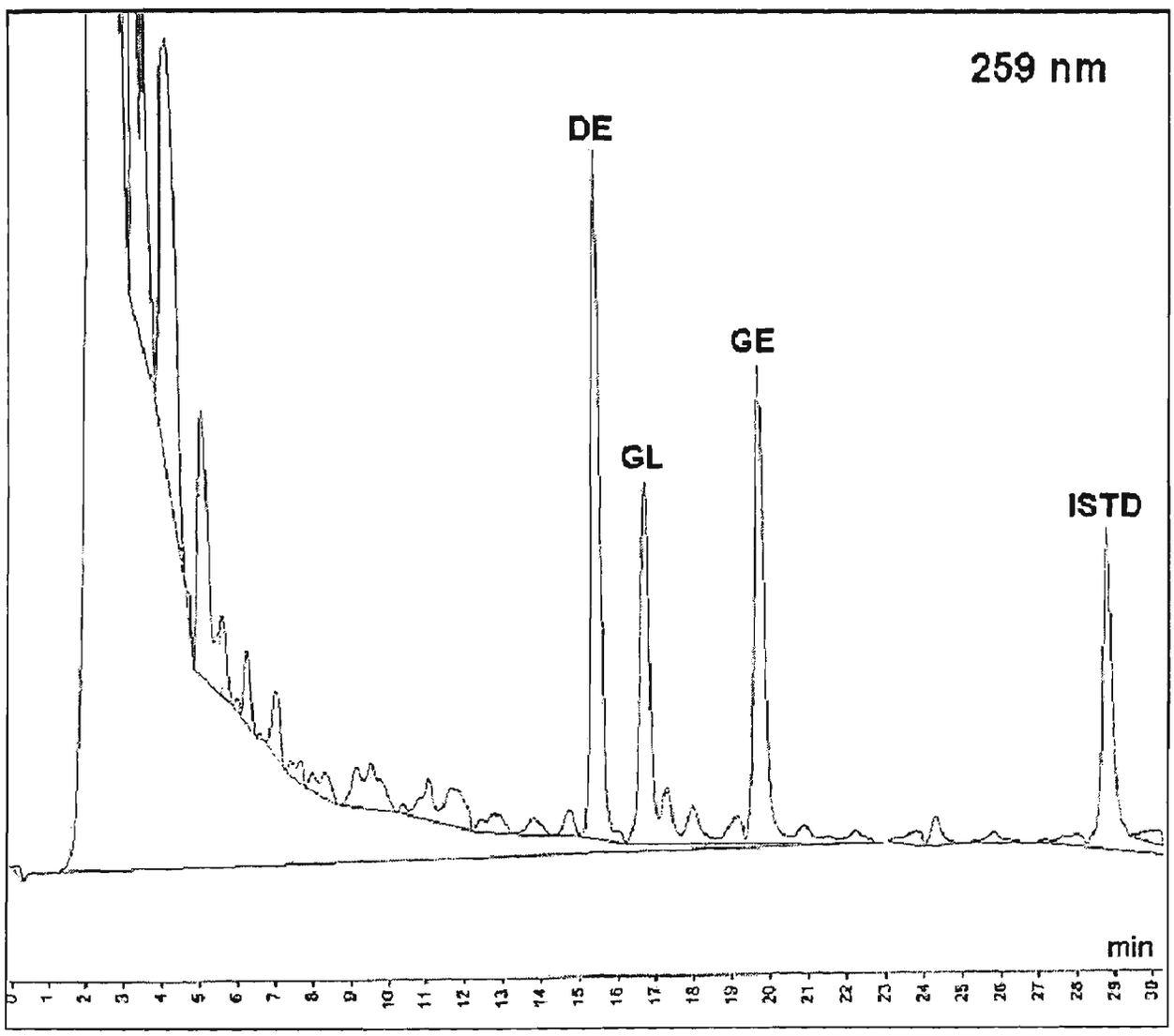

Figure 7.1 Reversed-phase high performance liquid chromatogram showing the approximate retention times of isoflavone isomers (detected at wavelength 259 nm) found in 24-hour pooled urine specimen; (DE) daidzein, 15.5 min; (GL) glycitein, 16.8 min; (GE) genistein, 19.8 min; (ISTD) benzophenone, 28.8 min.

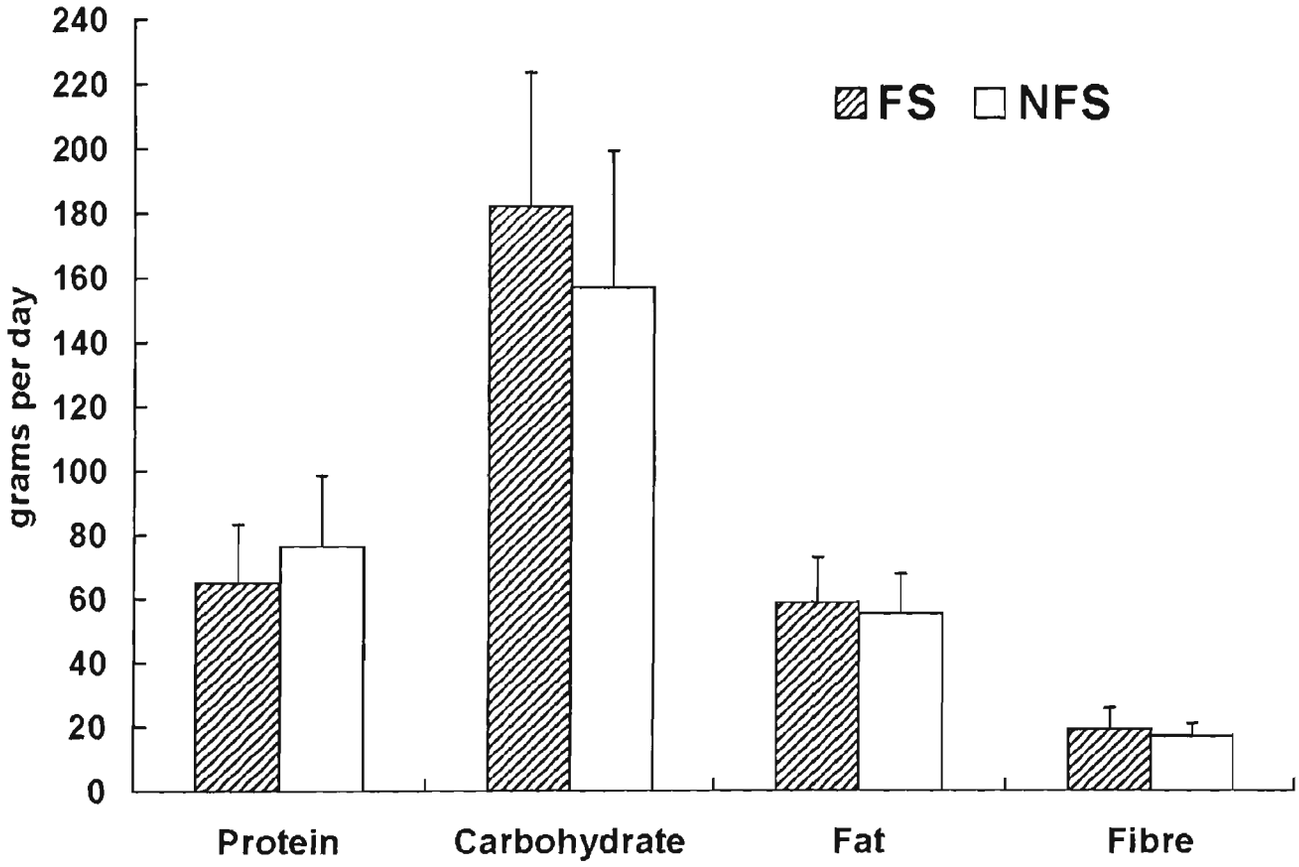

Figure 7.2 Daily intake of macronutrients (grams per day) for the fermented soymilk (FS) and non-fermented soymilk (NFS) group during supplementation. Fat intake represents the total of polyunsaturated, monounsaturated and saturated fats. Columns and error bars represent a mean ± standard deviation (n=24; eight subjects and three supplementation periods).

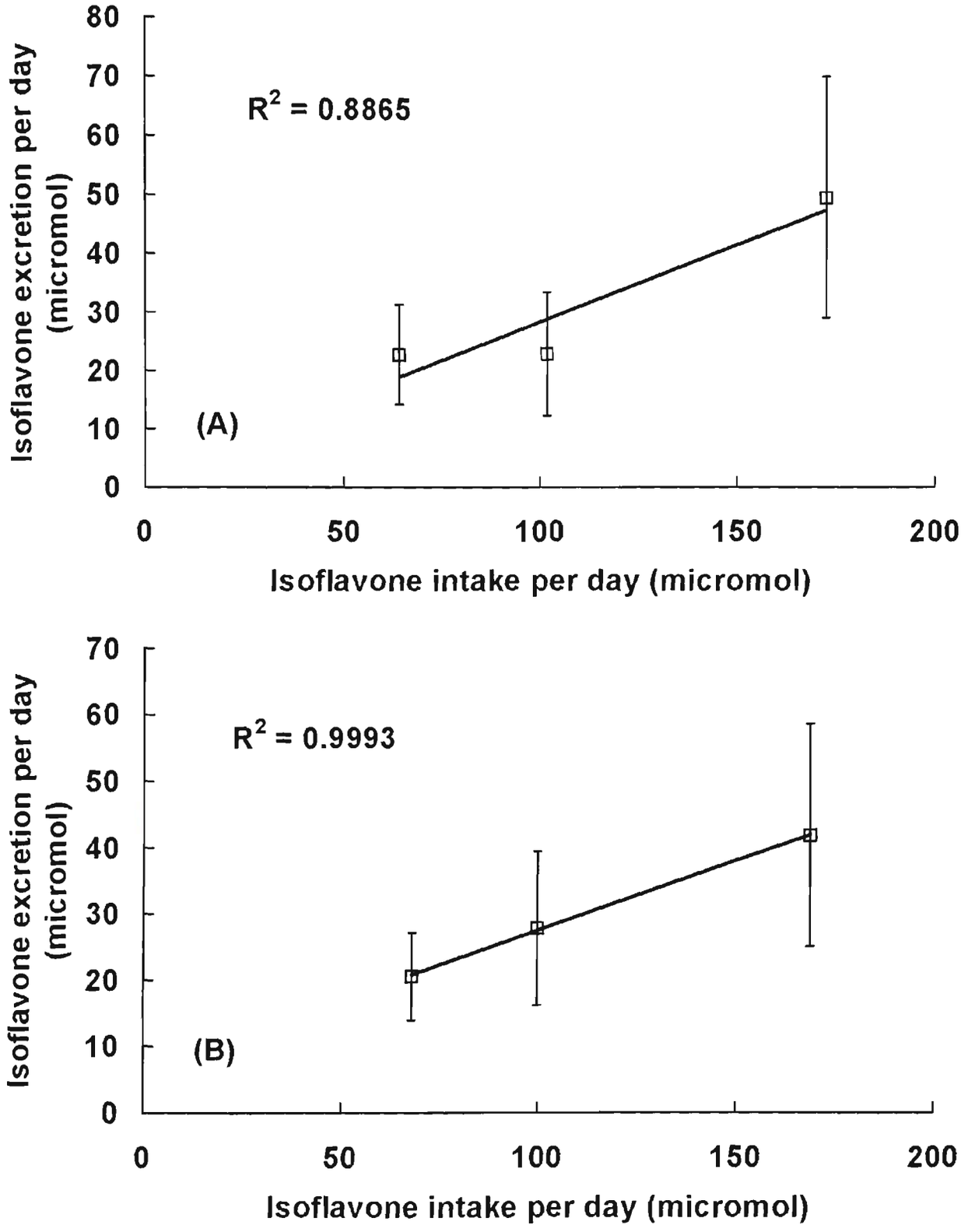

Figure 7.3 Relationship between the urinary excretion of isoflavone (total of daidzein, genistein and glycitein) versus the amount of isoflavone ingested per day during supplementation of non-fermented (A) and fermented (B) soymilk. Data points and error bars represent the mean ± standard deviation (n=48; eight subjects, three urine collection days and specimens analysed in duplicate). Solid lines represent the computed regression.

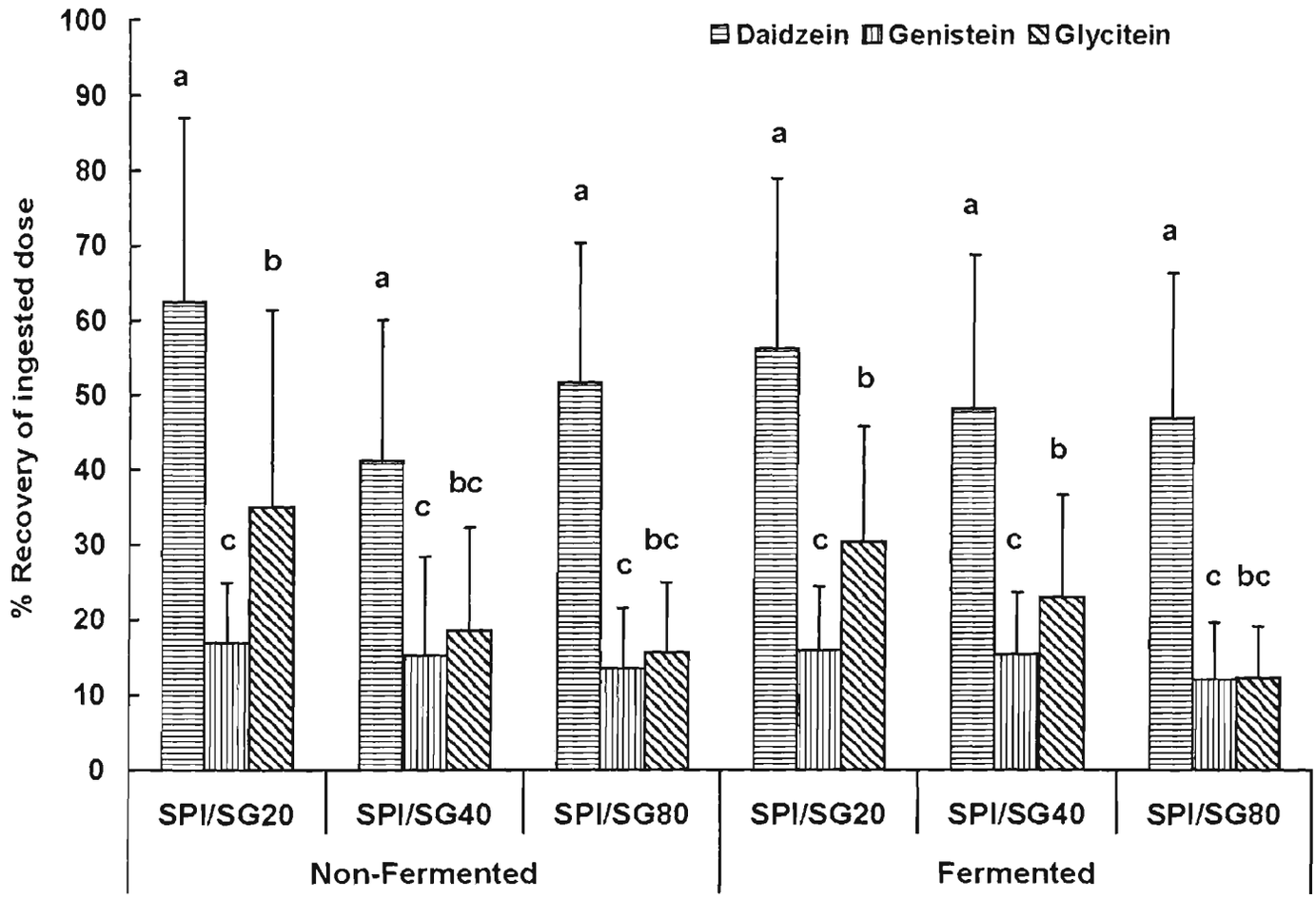

Figure 7.4 Ratio of the amount of daidzein, genistein and glycitein (total of conjugated and unconjugated forms) recovered in urine to the intake of each isomer per day (expressed as a percentage) from non-fermented and fermented soymilk. Columns and error bars represent a mean ± standard deviation (n=48; eight subjects, three urine collection days and specimens analysed in duplicate). Means of daidzein, genistein and glycitein arranged as three columns were analysed with one-way ANOVA. Columns with different letters are significantly different ($P<0.05$). SPI/SG20: Soymilk containing 20 mg isoflavone per 200 mL; SPI/SG40: Soymilk containing 40 mg isoflavone per 200 mL; SPI/SG80: Soymilk containing 80 mg isoflavone per 200 mL.

8.0 Urinary Excretion of Equol by Postmenopausal Women Consuming Soymilk Fermented by Bifidobacteria

8.1 INTRODUCTION

In recent clinical research on isoflavones, emphasis has once again been placed on the presence of equol in blood and urine and the ability of humans consuming soy foods to produce equol in the intestinal tract from its precursor daidzein and its glucosidic isomers. Equol has been singled out from other metabolites of daidzein and genistein because it has a structure similar to 17β-estradiol (Adlercreutz, 1990). Consequently, equol is suggested to be more oestrogenically active than daidzein (Price & Fenwick, 1985; Markiewicz *et al.* 1993; Rowland *et al.* 1999), having a greater affinity for ERs (Morito *et al.* 2001). Furthermore, Arora *et al.* (1998) found that equol was a more potent antioxidant than daidzein *in vitro*. However, clinical studies have shown that only 30 to 40% of men and women are able to synthesise equol in the intestinal tract in response to the ingestion of soy foods with a large inter-individual variability in the levels excreted in urine (Kelly *et al.* 1993; Hutchins *et al.* 1995; Lampe *et al.* 1998; Lu & Anderson, 1998; Slavin *et al.* 1998; Rowland *et al.* 2000; Setchell *et al.* 2003). The ability to produce equol appears to depend on the composition and enzymatic capability of gut microflora (Heinonen *et al.* 2002).

Several species of bacteria could be involved in the formation of equol in the intestinal tract (Turner *et al.* 2003). The genus *Bifidobacterium* is a predominant member of the intestinal microflora in the ileum and colon (Orrhage & Nord, 2000), the most probable site of equol synthesis (Setchell *et al.* 2003). Bifidobacteria possess complex enzyme systems which allow them to metabolise and structurally alter organic compounds during growth (Desjardins & Roy, 1990; Ballongue, 1993; Kamaly, 1997; Hou *et al.* 2000). In Chapters 3.0 to 6.0, it was reported that BB12 produced β-glucosidase and α-galactosidase which enabled them to hydrolyse isoflavone glucosides into aglycones and metabolise oligosaccharides

when grown in soymilk as well as breakdown aldehyde compounds. The formation of equol would require the reductive metabolism of daidzein by bifidobacteria, involving the removal of a carbonyl group and double bond from the pyran ring of the daidzein molecule. Dietary intake of carbohydrate and fat and its potential influence on bacterial metabolic activity in the intestinal tract has also been suggested to influence equol synthesis (Lampe *et al.* 1998; Slavin *et al.* 1998; Rowland *et al.* 2000). To date, studies on the formation and urinary excretion of equol have not investigated, in particular, the effect of consuming fermented soymilk comprising a greater proportion of daidzein in its free form and viable populations of bifidobacteria, which are able to modulate intestinal microbial balance and metabolic activity (Playne, 2002).

The objective of this study was to examine the urinary excretion of equol by postmenopausal women consuming either fermented soymilk, containing an enriched concentration of unconjugated daidzein and viable populations of BB12, or an isoflavone glucoside-rich non-fermented equivalent.

8.2 SUBJECTS AND METHODS

8.2.1 Subjects

The same sixteen healthy postmenopausal women involved in the study reported in Chapter 7.0 took part in this investigation and were recruited according to specific criteria described in section 7.2.1. Participants were randomly allocated to one of two groups to consume either fermented (FS group) or non-fermented (NFS group) soymilk during supplementation (section 7.2.1). The mean age and menopausal status of women in the FS and NFS group is also reported in section 7.2.1.

8.2.2 Study design

The study protocol was approved by the Human Research Ethics Committee of Victoria University and consisted of a randomised, double-blind, crossover design described in section 7.2.2. Each participant consumed a restricted but self-selected diet for the entire study and provided a 14-day weighed food and

beverage record during each supplementation period, as described in section 7.2.2. Daily energy, macronutrient and dietary fibre intake were quantified for each subject and the results reported in section 7.3.2 and Figure 7.2. Anthropometric measurements of height (m) and bodyweight (kg) were recorded and the BMI of each subject calculated (kg/m^2) before and after each supplementation period, (reported in section 7.3.1).

8.2.3 Collection and handling of urine specimens

Each participant collected four 24-hour pooled urine specimens per soymilk supplementation period; a day before beginning soymilk supplementation (baseline) and on days 4, 13 and 14. The same 24-hour pooled urine specimens analysed for isoflavones in Chapter 7.0 were analysed for equol in this study and were collected and processed in the manner described in section 7.2.3.

8.2.4 Soymilk ingredients

Pure culture of BB12 was obtained from the culture collection mentioned in section 3.2.1 and stored under the conditions described in section 4.2.1. SPI159 and SG were obtained from the suppliers mentioned in section 6.2.1. The macronutrient composition of SPI159 and SG (according to manufacturer specifications) is also described in section 6.2.1. The total concentration of daidzein and its glucosidic isomers in SPI159 and SG was 0.3 and 4.7 mg per gram, respectively (Table 6.1). Vanilla flavour (in propylene glycol) was obtained from the supplier mentioned in section 7.2.4.

8.2.5 Manufacture of soymilk

Three soymilk formulations were manufactured as described in section 7.2.5 and contained either 20 (SPI/SG20), 40 (SPI/SG40) or 80 (SPI/SG80) mg isoflavone per 200 mL serving. Forty-five bottles of non-fermented soymilk (200 mL each) of each formulation were prepared on three occasions (before each supplementation period of the NFS group) (section 7.2.5). A sample of 100 mL was taken

aseptically from three bottles of non-fermented soymilk (randomly selected from the batch) for the analysis of daidzein and its glucosides using HPLC (section 7.2.5).

8.2.6 Bacterial growth media

Rehydrated MRS agar (de Mann *et al.* 1960) containing additional 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine-HCl was prepared as described in section 3.2.2. Preparation of soymilk containing 4% (w/v) SPI159 and 1% (w/v) food-grade glucose powder, used for the activation of BB12, is described in section 7.2.6.

8.2.7 Fermentation of soymilk by bifidobacteria

On three occasions (before each supplementation period of the FS group) BB12 was activated and used in the production of 48 bottles (200 mL each) of fermented SPI/SG20, SPI/SG40 and SPI/SG80 as described in section 7.2.7. Three bottles were randomly taken from each batch of SPI/SG20, SPI/SG40 and SPI/SG80 at 0 and 24 h of incubation and a sample of 100 mL was withdrawn aseptically from each bottle for enumeration of viable BB12 populations (as described in section 3.2.6) and the remainder stored at –20°C. The viable populations of BB12 found in SPI/SG20, SPI/SG40 and SPI/SG80 (approximately 8 $\log_{10}$ CFU per mL) are reported in section 7.3.2. Frozen samples were freeze-dried as described in section 3.2.5 for the extraction and HPLC analysis of daidzein and its glucosidic isomers. The remaining 42 bottles of fermented soymilk (SPI/SG20, SPI/SG40 and SPI/SG80) were packaged into cartons comprising 14 bottles each and stored at 4°C prior to distribution.

8.2.8 HPLC apparatus, reagents and standards

Chromatographic analyses were carried out on a Varian® 9000 series high performance liquid chromatograph (instrument components mentioned in section 7.2.9) connected to an Exsil™ (SGE International) C18-ODS (250 x 4.6 mm internal diameter; 5 μm) reversed-phase column. HPLC grade

methanol and acetonitrile were purchased from Merck, and trifluoro-acetic acid, glacial acetic acid, sodium acetate and ammonium acetate from Sigma. All reagents used for the extraction of isoflavones and HPLC analyses were filtered through a 0.5 μm membrane (Millipore®) and mobile phases were degassed using nitrogen. Authentic standards of daidzein and benzophenone (ISTD) were obtained from Sigma, and standards of daidzin and equol from Indofine Chemical Co.

8.2.9 Extraction and HPLC analysis of daidzein and its glucosidic isomers in soymilk

The extraction of daidzein, daidzin, malonyldaidzin and acetyldaidzin from 1.50 g of freeze-dried SPI/SG20, 1.00 g of freeze-dried SPI/SG40 or 0.50 g of freeze-dried SPI/SG80 was performed in duplicate using the method described by Setchell *et al.* (2001). HPLC gradient elution was used to separate isoflavone isomers and the conditions are described in section 7.2.10. The photodiode array UV/VIS detector was set at 259 nm and the approximate retention times (min) of malonyldaidzin, daidzin, acetyldaidzin and daidzein are mentioned in section 7.2.10. Quantification of isoflavone isomers in soymilk using multi-level calibration and an ISTD was like that described in section 3.2.10. The concentrations of daidzein and its glucosidic isomers (expressed on a μmol per 200 mL basis) in non-fermented and fermented SPI/SG20, SPI/SG40 and SPI/SG80 are shown in Table 7.1.

8.2.10 Analysis of equol in urine specimens

The same aliquots of urine analysed for their daidzein, genistein and glycitein concentration in Chapter 7.0 were analysed for their equol concentration in this study. Equol was extracted from urine specimens using the method involving β-glucuronidase/sulfatase deconjugation described in section 7.2.11. HPLC conditions were as per Xu *et al.* (1994), with the photodiode array UV/VIS detector set at dual wavelengths of 282 nm (for the detection of equol) and 259 nm (for the detection of benzophenone). Six standards containing 20, 40, 50, 80, 100 and 200 ng of equol per 50 μL of methanol were used for the quantification of total urinary equol (conjugated and unconjugated forms). Twenty-microlitres of dimethyl sulfoxide were added to the crystalline form of equol to assist its dissolution in methanol.

Benzophenone (ISTD) was added to each equol standard at a concentration of 600 ng per 50 μL. The concentration of equol was calculated with respect to the ISTD and multiplied to represent the entire pooled urine specimen, according to the method of calculation described in Appendix F.

8.2.11 Statistical analysis

The urinary excretion of equol by each subject is represented as a mean ± standard deviation of six replicates (three 24-hour pooled urine specimens per supplementation period analysed in duplicate). For the FS and NFS group (each comprising eight subjects), concentrations of equol in 24-hour pooled urine specimens collected on day 4, 13 and 14 were analysed in duplicate and are presented as a mean ± standard deviation of sixteen replicates. To find significant differences in the urinary excretion of equol, means were analysed with ANOVA and 95% confidence intervals using Microsoft® Excel Stat Pro™ as described by Albright *et al.* (1999). ANOVA data with a $P<0.05$ was classified as statistically significant (two-sided test).

8.3 RESULTS AND DISCUSSION

8.3.1 HPLC analysis of equol in urine

The reversed-phase HPLC method used in this study to quantify equol in urine specimens was adapted from two earlier methods devised by Franke & Custer (1994) and Xu *et al.* (1994), with the latter a modified version of a method by Lundh *et al.* (1988). Methods by Lundh *et al.* (1988) and Franke & Custer (1994) were similar in that they used UV/VIS detection of equol (at its UV absorption maximum of 280 nm) after enzymatic hydrolysis of isoflavone metabolites by glucuronidase/sulfatase. However, the method by Franke & Custer (1994) showed far greater sensitivity for the detection of equol. Lundh *et al.* (1988) reported that they were able to detect a concentration of 4000 ng of equol per mL of urine (17 μmol per L), whereas Franke & Custer (1994) detected 151 ng of equol per mL (0.62 μmol per L). The HPLC-UV/VIS method used in this study was able to detect 0.41 μmol equol per L of urine, closer to the detection limit reported by Franke & Custer (1994). Equol standards below a concentration of 50

ng per 50 μL (injection volume) were not detected using the HPLC-UV/VIS method described in section 8.2.10, limiting the accuracy of quantifying this compound in urine. The retention time of equol, at a wavelength of 282 nm, was approximately 17.3 min (Figure 8.1), between isoflavone isomers of glycitein (16.8 min) and genistein (19.8 min) detected at their UV/VIS absorption maximum of 259 nm (Figure 7.1).

Franke *et al.* (1998) reported that low concentrations of equol in urine were quantified less accurately than other isoflavone metabolites using HPLC-UV/VIS, with a detection limit that was 3 and 8 times higher than that of daidzein and genistein, respectively. According to Hendrich (2002), HPLC with UV/VIS detection is the least sensitive method currently used for the quantification of equol in body fluids, further stating that HPLC with coulometric detection (Gamache & Acworth, 1998) is probably the method of choice, being less expensive and easier to institute than the gas chromatography-mass spectroscopy (Adlercreutz *et al.* 1991; Heinonen *et al.* 1999) or immunofluorescence methods (Uehara *et al.* 2000) and being more sensitive than HPLC-UV/VIS methods of similar cost. Gamache & Acworth (1998) reported that the detection limit for equol analysed using HPLC with coulometric detection was 1 ng per mL urine (0.0041 μmol per L), approximately 100 times more sensitive than the HPLC-UV/VIS method used in this study. However, HPLC-UV/VIS instrumentation is probably the most common and widely available of those analytical instruments mentioned above; hence, they are still used to quantify equol and other isoflavone metabolites despite their lack of sensitivity (Lundh *et al.* 1988; Franke & Custer, 1994; Xu *et al.* 1994, 1995, 2000; Franke *et al.* 1995, 1998).

8.3.2 Equol producers versus non-producers

Due to the potential importance of equol formation as a result of soy food consumption and its possible positive effects on human health, previous studies on the bioavailability of isoflavones and the urinary excretion of isoflavone metabolites have dichotomised subjects into equol producers (or excreters) and non-producers (or non-excreters) (Setchell *et al.* 1984; Adlercreutz *et al.* 1991; Kelly *et al.* 1993;

Hutchins *et al.* 1995; Slavin *et al.* 1998; Rowland *et al.* 2000). Recent clinical studies by Uchiyama *et al.* (2001) and Lydeking-Olsen *et al.* (2002) have supported the classification of soy consumers into either equol producers or non-producers, by discovering that subjects able to produce equol after consuming soy foods showed greater benefits where hormone-sensitive end points were concerned. Early studies by Setchell *et al.* (1984) and Adlercreutz *et al.* (1991) classified subjects as equol producers if their equol excretion increased significantly over baseline values in response to a soy challenge or their urinary equol concentrations differed significantly from zero while they consumed their habitual diets, respectively. As a result, studies by Setchell *et al.* (1984) and Adlercreutz *et al.* (1991) showed that 67% of subjects were equol producers. This broad definition of an equol producer was refined by Kelly *et al.* (1993) into low-equol (< 2.6 μmol per day) and high-equol (> 8.3 μmol per day) excreters, stating that most subjects excrete detectable amounts of equol while consuming their habitual diets even if soy is not a normal component of that diet. In support, King *et al.* (1998) found low concentrations of equol in cow's milk (0.021 to 0.12 μmol per 100 mL), which is a commonly consumed beverage amongst Western peoples. Cows derive the daidzein precursor formononetin from clover, which may be transformed to equol via ruminal microflora. In our study, we decided to classify a subject as an equol producer if their urinary excretion of equol was > 1 μmol per day, based on the definition established by Rowland *et al.* (2000) and similar to that of Hutchins *et al.* (1995) (> 1.5 μmol per day).

The mean urinary excretion of equol by each subject in the NFS and FS group during supplementation of SPI/SG20, SPI/SG40 and SPI/SG80 is shown in Table 8.1. Considering that the detection limit for equol was high at 0.41 μmol per L of urine, we still found six women in the NFS group (75%) and four women in the FS group (50%) who excreted detectable levels of equol during one or more of the supplementation periods. However, of these ten women who excreted equol during soymilk supplementation, none were classified as an equol producer (urinary equol excretion of > 1 μmol per day) at each isoflavone dosage level (SPI/SG20, SPI/SG40 and SPI/SG80) according to the mean

concentrations of urinary equol shown in Table 8.1. Nevertheless, two women from both the NFS and FS group (25% of each group) were classified as equol producers during at least one of the soymilk supplementation periods. In the NFS group, there was an equol producer during supplementation of the lowest and highest dosage of daidzein and its glucosides (SPI/SG20 and SPI/SG80), whereas in the FS group equol producers were found during the ingestion of only the higher dosages of daidzein and its glucosides (SPI/SG40 and SPI/SG80). Subject 4 of the NFS group and subject 6 of the FS group excreted significantly greater levels of equol during supplementation of SPI/SG20 (P=0.003) and SPI/SG40 (P=0.009), respectively. For subject 8 of the FS group, a combination of a greater dosage of unconjugated daidzein derived from fermented SPI/SG80 (Table 7.1) together with the ingestion of viable populations of BB12 appeared to stimulate the formation and excretion of equol. Subject 8 of the FS group excreted 1.17 μmol equol per day during supplementation of SPI/SG80, but did not excrete detectable levels of equol during supplementation of fermented SPI/SG20 and SPI/SG40 (Table 8.1). However, the greatest dosage of daidzein and its glucosidic isomers via non-fermented SPI/SG80 also appeared to enhance the excretion of equol by subject 1 of the NFS group. In this case, it was not possibly related to the ingestion of a higher proportion of unconjugated daidzein and viable BB12 populations. Hence, a high dose of daidzein at 67 μmol per day (via SPI/SG80) potentially stimulated the formation and urinary excretion of equol by two subjects who did not excrete detectable levels of equol at lower dosages, regardless of whether the concentration of daidzein was found in an aglycone- or glucoside-rich configuration.

8.3.3 Urinary excretion of equol during supplementation of non-fermented and fermented soymilk

Each subject consumed three daily dosages of the equol precursor daidzein (total of unconjugated and glucoside forms) at 22.6, 37.4 and 67.3 μmol per day via SPI/SG20, SPI/SG40 and SPI/SG80, respectively. The specific concentrations of unconjugated daidzein and its glucoside isomers of daidzin, malonyldaidzin and acetyldaidzin in each of SPI/SG20, SPI/SG40 and SPI/SG80 are shown in Table

7.1. Women in the NFS group consumed non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 comprising 16% (3.4 μmol per 200 mL serving), 13% (4.8 μmol per 200 mL serving) and 9% (6.2 μmol per 200 mL serving) of the total concentration of daidzein in an unconjugated aglycone configuration, respectively (Table 7.1). Fermentation of SPI/SG20, SPI/SG40 and SPI/SG80 by BB12 significantly increased the proportion of aglycone structures to 73% (17.0 μmol per 200 mL serving), 63% (23.8 μmol per 200 mL serving) and 43% (29.0 μmol per 200 mL serving) of total daidzein, respectively, which was ingested by women in the FS group (Table 7.1). No previous studies have investigated whether the formation and excretion of equol by a group of postmenopausal women is enhanced by the ingestion of a greater proportion of the precursor daidzein in its free form via soymilk fermented by bifidobacteria.

The mean urinary excretion of equol (total of conjugated and unconjugated forms) by the FS and NFS group (each n = 8) at day 4, 13 and 14 of soymilk supplementation is shown in Table 8.2. On the whole, mean urinary excretion of equol by the FS and NFS group (including equol producers, non-producers and subjects who did not excrete detectable levels of equol) ranged between 0.06 and 0.63 μmol per day, with no equol detected in any of the baseline urine specimens of both groups. Furthermore, there was a trend toward a positive correlation between urinary equol excretion and dosage level of daidzein and its glucosides, with greater urinary excretion of equol shown during supplementation of SPI/SG80 (Table 8.2). Rowland *et al.* (2000) found that 23 subjects ingesting a high dosage of daidzein and its glucosides (84 μmol per day) excreted an average of 3.9 μmol equol per day, 92 times the level excreted by subjects (n = 21) ingesting a dosage of 3.5 μmol daidzein per day. The distinctly different dosage levels of daidzein and its glucosides between the two groups in the study by Rowland *et al.* (2000) resulted in an obvious positive correlation between equol excretion and dosage level. In our study, even though the greatest urinary excretion of equol occurred during supplementation of fermented and non-fermented SPI/SG80, it was not significantly greater than the level of equol excreted at lower dosages of daidzein and its glucosides via SPI/SG20 and SPI/SG40 ($P>0.05$) (Table 8.2). This

is probably due to the considerable inter-individual variation in equol excretion within each group as represented by the large values of standard deviation (Table 8.2). The absolute urinary excretion of daidzein was also highest during supplementation of SPI/SG80 (Table 7.3), even though this dosage was associated with the lowest percentage recovery of daidzein (Figure 7.4). As discussed previously (section 7.3.4), only a limited amount of a high dose of daidzein (via SPI/SG80) may be absorbed proximally in the intestinal tract, with the remaining fraction passing into the ileum and colon where there are greater populations of intestinal bacteria that could biotransform daidzein into equol and other metabolites. Hence, this may be reason for the greater levels of equol excreted during ingestion of SPI/SG80. However, there were no significant differences in urinary equol excretion between the NFS and FS group at any of the dosage levels ($P>0.05$), to indicate that the consumption of soymilk fermented by bifidobacteria enhanced the formation and excretion of equol amongst a group of postmenopausal women (Table 8.2). Similarly, Hutchins *et al.* (1995) reported that women ingesting a greater proportion of daidzein in an unconjugated form via fermented soy food (tempeh) did not produce and excrete higher levels of equol than women consuming isoflavone glucoside-rich cooked soybean pieces.

In earlier studies that examined the urinary excretion of equol, Xu *et al.* (1994, 1995) did not detect equol in urine collected from women during the 24 hours after the administration of either a single dose of soymilk or three doses of soymilk on a single day. More recently, both Richelle *et al.* (2002) and Setchell *et al.* (2003) reported that there was some delay of up to 24 h before equol appeared in urine. In our study, pooled urine collection was allocated to days 4, 13 and 14 of soymilk supplementation, for adaptation of the gut microflora to the ingestion of soymilk, and for the intestinal microbial balance of women in the FS group to change as a result of the consumption of viable BB12 populations. As shown in Table 8.2, in every case except during the supplementation of non-fermented SPI/SG80, mean urinary equol excretion for the FS and NFS group tended to be greater after 13 and 14 days of soymilk supplementation compared to the excretion on day 4, however, there was no significant difference

observed between these time points at any of the dosage levels (P>0.05). According to Hendrich (2002), the ability to produce equol is dependent on adaptation of gut microflora to the presence of daidzein in the diet. Lu & Anderson (1998) reported that some women who were initially unable to metabolise daidzein into equol, appeared to develop this ability over a month of daily soy ingestion. Similarly, subject 1 and 2 of the FS group, who both excreted detectable levels of equol during supplementation of SPI/SG20, SPI/SG40 and SPI/SG80 (represented as mean of day 4, 13 and 14; Table 8.1), did not excrete detectable levels of equol on day 4. However, on day 13 and 14 of soymilk supplementation, subject 1 and 2 of the FS group excreted > 1 μmol equol, which ultimately classified them both as equol producers (as described in section 8.3.2). As a result, the FS group comprised a total of four equol producers (50%), two of whom exhibited a delayed response to the ingestion of daidzein. None of the other women in the FS and NFS group showed a delayed excretion of equol. On the whole, six women in our study (38% of total participants) excreted > 1 μmol equol per day, and this proportion of equol producers was similar to that reported by Rowland *et al.* (2000). In their study, 36% of the study group were equol producers.

Similar to the results of Hutchins *et al.* (1995), there was no evidence of a correlation between BMI, age and the ratio of dietary fat, carbohydrate and fibre intake and the excretion of equol amongst the equol producers identified in Table 8.1. Each had a similar dietary fat, carbohydrate and fibre intake to the non-producers in their respective supplementation groups (P>0.05) (the mean daily intake of macronutrients by the FS and NFS group are shown in Figure 7.2). Rowland *et al.* (2000) reported that a high intake of dietary fat decreased the capacity of gut microflora to transform daidzein into equol, whereas a high intake of carbohydrates enhanced equol excretion in women. Subject 1 and 2 of the FS group, who both showed a delay in the excretion of equol (as described above), had daily intakes of carbohydrate and fibre that may have enhanced the ability of their intestinal microflora to biotransform daidzein into equol. Of the subjects in the FS group, subject 1 had the highest intake of carbohydrate and fibre during each supplementation period (SPI/SG20, SPI/SG40 and SPI/SG80), at approximately

225 g per day (51% of energy) and 29 g per day, respectively. Subject 2 of the FS group had the second highest intake of carbohydrate during each fermented soymilk supplementation period, at approximately 203 g per day (42% of energy), but a fibre intake that was similar to the average of the group. Rowland *et al.* (2000) reported that good equol producers consumed a higher proportion of energy as carbohydrate (55 ± 2.9%) than poor equol producers. In addition, Rowland *et al.* (2000) stated that good equol producers consumed a lower proportion of energy as fat than poor producers (26 ± 2.3% compared with 35 ± 1.6%). In contrast, even though subject 2 of the FS group had the second highest intake of carbohydrate, which may have enhanced the intestinal formation of equol, this subject also had the highest daily intake of fat during each fermented soymilk supplementation period, at approximately 80 g per day (38% of energy). In our study, the association between daily macronutrient intake and the excretion of equol by subject 1 and 2 of the FS group may have been influenced by the daily ingestion of viable populations of BB12, able to adjust intestinal microbial balance and metabolic activity more rapidly and effectively than the ingestion of fermentable substrates alone. Furthermore, there may have been a synergistic relationship between the ingestion of viable BB12 via fermented soymilk and greater total intake of carbohydrate and fibre (per day) that stimulated the growth and metabolic activity of intestinal bacteria in subject 1 and 2 of the FS group, enhancing the excretion of equol to > 1 μmol on days 13 and 14 of their soymilk supplementation. Hence, the ingestion of viable populations of BB12 may not be enough to enhance the metabolism of daidzein into equol and may need to be consumed alongside a high dietary intake of carbohydrate and fibre (50 to 60% of energy intake).

The percentage of the ingested dose of daidzein (including unconjugated and conjugated isomers) via non-fermented and fermented SPI/SG20, SPI/SG40 and SPI/SG80 recovered as urinary equol is shown in Figure 8.2. The percentage of daidzein recovered as equol ranged from 0.6% to 1.6%, with supplementation of the lowest dosages of daidzein from fermented and non-fermented SPI/SG20 showing greater urinary percentage recovery of equol than the ingestion of SPI/SG40 and SPI/SG80

(P=0.18). In comparison, the urinary percentage recovery of daidzein in its aglycone configuration ranged from 41% to 63% (Figure 7.4). There appeared to be a similar pattern in the urinary recovery of both daidzein (Figure 7.4) and equol (Figure 8.2), with urinary percentage recovery inversely related to dosage level. With respect to the urinary percentage recovery of daidzein, it was suggested in Chapter 7.0 (section 7.3.4) that a lower dose of daidzein may be absorbed more effectively in the proximal gastrointestinal tract, reflecting more complete and rapid absorption. However, with regard to equol, because the greatest levels of equol excreted by subjects were in the range of 1.1 to 1.4 μmol per day and found at each of the dosage levels (Table 8.1), we believe this evidence does not support the inverse correlation between percentage recovery of equol and dosage level indicated in Figure 8.2. Furthermore, the ingestion of fermented soymilk appeared to have no effect on urinary excretion of equol, with no significant difference in percentage recovery observed between the NFS and FS group at each dosage level of daidzein and its glucosides (P>0.05) (Figure 8.2).

Each of the fermented soymilks contained viable populations of BB12 at approximately 8 $\log_{10}$ CFU per mL, which are able to effectively modulate intestinal microbial balance in favour of other *Bifidobacterium* species and enzyme-producing probiotic bacteria in the intestinal tract (Playne, 2002). From previous studies, it is not known whether inter-individual variation in equol formation and urinary excretion can be reduced by establishing a more consistent intestinal microflora amongst subjects via the ingestion of probiotic bifidobacteria. According to the values of standard deviation shown in Table 8.2, inter-individual variation within the FS group appeared to be equivalent to or even greater than that observed in the NFS group, with the modulation of intestinal microbial balance having little or no effect on reducing inter-individual variation in equol formation and excretion.

8.4 CONCLUSIONS

As a group, women ingesting fermented soymilks did not excrete greater amounts of equol during supplementation or have greater urinary recovery of equol than women consuming non-fermented

soymilks. Furthermore, the ingestion of viable populations of BB12, at levels able to modulate intestinal microbial balance, did not appear to reduce inter-individual variation in the urinary excretion of equol amongst postmenopausal women consuming fermented soymilks when compared to the non-fermented soymilk group. Nevertheless, of the six women classified as equol producers (representing about a third of the entire study group), four were from the fermented soymilk supplementation group. Amongst the equol producers, various conditions appeared to stimulate the formation and excretion of equol at levels > 1 μmol per day, indicating that it may be a combination of factors, including dosage level of daidzein, the composition of daidzein and its glucosidic isomers and/or dietary macronutrient intake. For two of the equol producers, the highest dosage of daidzein and its glucosides (67 μmol per day) was needed to stimulate the formation and excretion of equol to > 1 μmol per day, regardless of whether daidzein was ingested in an aglycone-rich (fermented soymilk) or glucoside-rich (non-fermented soymilk) configuration. For two other equol producers, who showed a delay in their urinary excretion of equol, it was the ingestion of a high proportion of unconjugated daidzein and viable populations of BB12 (via fermented soymilk) together with a high dietary intake of carbohydrate and fibre that appeared to stimulate equol formation. Consistent with the findings of earlier studies, there appeared to be an underlying factor(s) which influenced whether or not these women had the capacity to metabolise daidzein into equol. Hence, even though the consumption of fermented soymilk comprising up to 60 μmol daidzein per serving may have stimulated the formation of equol in postmenopausal women who were potential equol producers, there was no strong evidence to suggest it could instigate the biotransformation of daidzein into equol in those women predetermined as non-producers.

Table 8.1 Urinary excretion of equol[1] (μmol per day[2]) by each subject in the non-fermented soymilk (NFS) and fermented soymilk (FS) group during supplementation of SPI/SG20, SPI/SG40 and SPI/SG80 (mean ± standard deviation; n = 6[3])

Group	Soymilks		
Subject no.	SPI/SG20	SPI/SG40	SPI/SG80
NFS			
1[4]	ND	ND	1.41 ± 0.54^{A}
2	ND	ND	ND
3	ND	ND	ND
4[4]	$1.12\pm0.06^{a,A}$	$0.62\pm0.09^{bc,A}$	$0.50\pm0.21^{c,A}$
5	ND	0.40±0.36	ND
6	$0.61\pm0.53^{a,A}$	$0.61\pm0.08^{a,A}$	$0.44\pm0.03^{a,A}$
7	ND	ND	0.67 ± 0.10^{A}
8	$0.88\pm0.08^{a,A}$	$0.33\pm0.29^{b,A}$	$0.65\pm0.15^{ab,A}$
FS			
1	$0.84\pm0.78^{a,A}$	$0.97\pm0.85^{a,A}$	$0.77\pm0.56^{a,A}$
2	$0.80\pm0.77^{a,A}$	$0.79\pm0.69^{a,A}$	$0.88\pm0.77^{a,A}$
3	ND	ND	ND
4	ND	ND	ND
5	ND	ND	ND
6[4]	$0.48\pm0.05^{bc,A}$	$1.16\pm0.07^{a,A}$	$0.41\pm0.36^{c,A}$
7	ND	ND	ND
8[4]	ND	ND	1.17 ± 0.53^{A}

SPI/SG20: Soymilk containing 23 μmol of daidzein and its glucosides per 200 mL; SPI/SG40: Soymilk containing 37 μmol of daidzein and its glucosides per 200 mL; SPI/SG80: Soymilk containing 67 μmol of daidzein and its glucosides per 200 mL.
ND: Equol not detected in any of the urine specimens analysed in duplicate using a 20 mL aliquot and injection volume of 50 μL (detection limit of 0.41 μmol per L).
Means in the same row and column with different lower- and upper-case superscript, respectively, are significantly different ($P<0.05$).
[1]Concentration of equol after β-glucuronidase/sulfatase deconjugation (conjugated and unconjugated forms).
[2]Concentration of equol in 24-hour pooled urine specimen.
[3]Three 24-hour pooled urine specimens (collected on days 4, 13 and 14 of supplementation) analysed in duplicate.
[4]Excreted a mean concentration of > 1 μmol equol per day during at least one of the supplementation periods and was subsequently classified as an equol producer.

Table 8.2 Urinary excretion of equol[1] (μmol per day[2]) by 2 groups of 8 women[3] during supplementation of fermented and non-fermented SPI/SG20, SPI/SG40 and SPI/SG80 (mean ± standard deviation; n = 16[4])

Day of 24-hour pooled urine collection	Non-Fermented			Fermented		
	SPI/SG20	SPI/SG40	SPI/SG80	SPI/SG20	SPI/SG40	SPI/SG80
Baseline[5]	ND	ND	ND	ND	ND	ND
Day 4	$0.25\pm0.47^{a,A}$	$0.13\pm0.25^{a,A}$	$0.53\pm0.62^{a,A}$	$0.06\pm0.18^{a,A}$	$0.14\pm0.38^{a,A}$	$0.16\pm0.31^{a,A}$
Day 13	$0.36\pm0.51^{a,A}$	$0.33\pm0.35^{a,A}$	$0.43\pm0.52^{a,A}$	$0.29\pm0.43^{a,A}$	$0.47\pm0.65^{a,A}$	$0.42\pm0.52^{a,A}$
Day 14	$0.42\pm0.48^{a,A}$	$0.27\pm0.29^{a,A}$	$0.42\pm0.37^{a,A}$	$0.44\pm0.69^{a,A}$	$0.49\pm0.69^{a,A}$	$0.63\pm0.75^{a,A}$

SPI/SG20: Soymilk containing 23 μmol of daidzein and its glucosides per 200 mL; SPI/SG40: Soymilk containing 37 μmol of daidzein and its glucosides per 200 mL; SPI/SG80: Soymilk containing 67 μmol of daidzein and its glucosides per 200 mL.

ND: Equol not detected in any of the urine specimens analysed in duplicate using a 20 mL aliquot and injection volume of 50 μL (detection limit of 0.41 μmol/L).

Means in the same row and column with different lower- and upper-case superscript, respectively, are significantly different ($P<0.05$).

[1]Concentration of equol after β-glucuronidase/sulfatase deconjugation (conjugated and unconjugated forms).

[2]Concentration of equol in 24-hour pooled urine specimen.

[3]Consisting of two separate groups consuming either each of the fermented or non-fermented soymilks.

[4]Eight 24-hour pooled urine specimens (one per subject) analysed in duplicate.

[5]Total concentration of equol in 24-hour pooled urine specimen collected one day before soymilk supplementation.

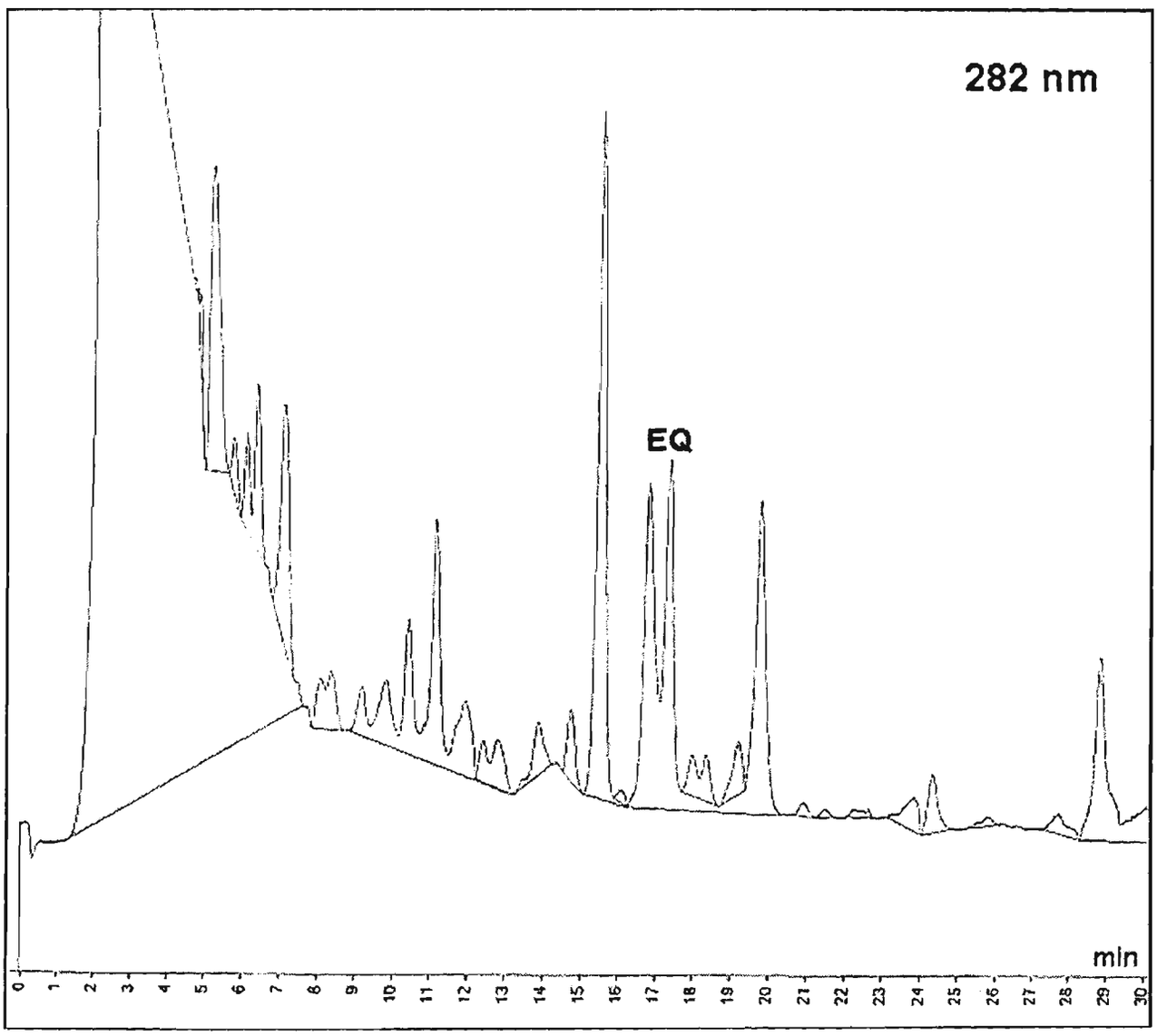

Figure 8.1 Reversed-phase high performance liquid chromatogram showing the approximate retention time of equol (detected at wavelength 282 nm) found in 24-hour pooled urine specimen; (EQ) equol, 17.3 min.

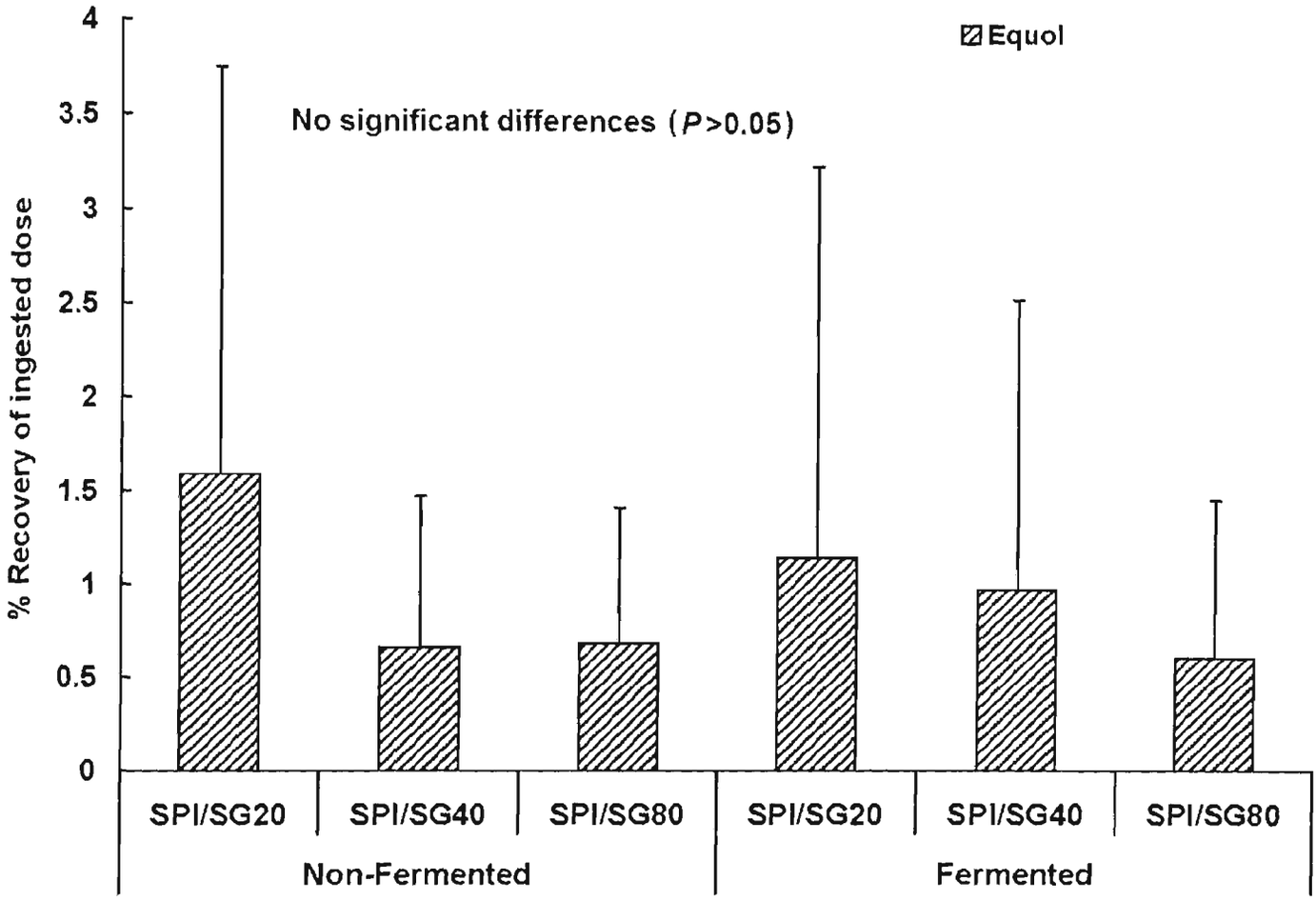

Figure 8.2 Ratio of the amount of equol (total of conjugated and unconjugated forms) recovered in urine to the intake of its precursor daidzein and its glucosidic conjugates per day (expressed as a percentage) from non-fermented and fermented soymilk. Columns and error bars represent a mean ± standard deviation (n=48; eight subjects, three urine collection days and specimens analysed in duplicate). Means were analysed with one-way ANOVA. SPI/SG20: Soymilk containing 23 μmol of daidzein and its glucosides per 200 mL; SPI/SG40: Soymilk containing 37 μmol of daidzein and its glucosides per 200 mL; SPI/SG80: Soymilk containing 67 μmol of daidzein and its glucosides per 200 mL.

9.0 Composite Effects of Isoflavones and Bifidobacteria on Lipid Profiles, Bone Turnover and Sex Steroid Hormones in Postmenopausal Women

9.1 INTRODUCTION

Findings from earlier clinical studies on the potential positive effects of isoflavones on cardiovascular disease risk factors [lipid profiles and Lp(a)], bone turnover markers, and hormone profiles (FSH, LH and SHBG) in postmenopausal women have been highly variable and inconclusive. Apart from the differences in protocol between these clinical trials, in terms of the size of the study group, duration of treatment, study design (parallel versus crossover), and isoflavone dosage levels (in the range of 10 to 165 mg per day), the vehicle for administering isoflavone has also differed considerably. Some studies have administered isoflavone in the form of pure isoflavone tablets (Hodgson *et al.* 1998; Simons *et al.* 2000; Dewell *et al.* 2002) or isoflavone extracts (Uesugi *et al.* 2002). However, the majority of clinical trials have involved the consumption of soy foods, including soy protein (Baum *et al.* 1998; Potter *et al.* 1998; Duncan *et al.* 1999; Alekel *et al.* 2000; Wangen *et al.* 2000, 2001; Gardner *et al.* 2001; Teede *et al.* 2001; Jenkins *et al.* 2002; Nicholls *et al.* 2002; Persky *et al.* 2002), soybeans (Baird *et al.* 1995), and soymilk (Scheiber *et al.* 1999; Pino *et al.* 2000). With respect to cardiovascular disease risk, postmenopausal women ingesting soy protein have shown some improvements in their lipid profiles (Baum *et al.* 1998; Scheiber *et al.* 2001; Jenkins *et al.* 2002). On the other hand, clinical studies by Baird *et al.* (1995), Duncan *et al.* (1999), Pino *et al.* (2000), Persky *et al.* (2002) and Nicholls *et al.* (2002) found no strong evidence to indicate that isoflavones derived from soy foods have a positive influence on hormone profiles (LH, FSH and endogenous oestrogens) in postmenopausal women, other than some evidence to indicate that isoflavone intake increases concentrations of SHBG. Nevertheless, Scheiber *et al.* (1999) reported that postmenopausal women consuming soymilk providing 60 to 70 mg isoflavone per day showed significant reductions in bone resorption marker N-telopeptide and increases in bone formation marker serum osteocalcin after 12 weeks of treatment.

The form in which isoflavone is ingested may influence its physiological effects on biomarkers of disease risk in postmenopausal women, considering the importance of intestinal microflora in the absorption and ultimate bioactivity of isoflavones (Heinonen *et al.* 2002; Hendrich, 2002; Turner *et al.* 2003). Previous clinical studies on postmenopausal women have provided isoflavones in a glucoside-rich configuration, typically via a non-fermented product, possibly causing the large inter-individual variation in physiological response to isoflavone treatment due to the proposed influence of intestinal microflora on isoflavone bioavailability (Hendrich, 2002). As reported in Chapter 7.0, even though the ingestion of fermented soymilk comprising a greater proportion of isoflavone in an aglycone form did not enhance the urinary excretion or percentage recovery of isoflavone, it did appear to reduce inter-individual variation in isoflavone excretion shown by a distinct linear dose-response (Figure 7.3). The high linearity in isoflavone dose-response may have also been due to the modulation of intestinal microbial balance as a result of ingesting viable bifidobacteria, establishing a more consistent microflora amongst postmenopausal women consuming fermented soymilk (Chapter 7.0). In addition, *in vitro* and clinical studies have shown that *Bifidobacterium* sp. exert positive effects on the health of the host upon ingestion, including a possible cholesterol-lowering effect (section 2.6.3). Hence, soymilk containing a greater proportion of isoflavone in an aglycone configuration and viable populations of BB12 may be of greater health benefit to postmenopausal women.

The objective of this study was to examine the effects of ingesting an isoflavone aglycone-enriched fermented soymilk containing viable bifidobacteria on serum lipid profiles and Lp(a), bone resorption, and levels of FSH, LH and SHBG in postmenopausal women.

9.2 SUBJECTS AND METHODS

9.2.1 Subjects

Thirty-six healthy postmenopausal women, recruited from the Melbourne metropolitan area from 122 interested volunteers, completed all the requirements of the study. Seventeen women withdrew from the study for unforeseen reasons. Each volunteer was interviewed and screened using a health information and food frequency questionnaire (Appendix C) designed to exclude those who had:

gastrointestinal disorders or diseases of the cardiovascular, systemic or endocrine systems; food allergies; an alcohol intake greater than two standard drinks per day; a smoking habit; regularly used prescription or non-prescription medication; used antibiotics in the past 3 months or ERT in the past 6 months; or had dietary patterns not representative of the general population (e.g. strict vegetarian, consumption of more than two servings of soy food per week). Participants were asked to read a plain language statement outlining the reasons for and requirements of the study, and sign a consent form (Appendix D). They were then randomly allocated to one of three groups, to consume either fermented soymilk, non-fermented soymilk or a placebo of milk casein. At the end of the study, the fermented soymilk (FS) and non-fermented soymilk (NFS) group each consisted of 13 subjects and the casein-milk (CAS) group consisted of 10 subjects. The mean (± standard deviation) age of women in the FS, NFS and CAS group was 57.5 (± 3.8), 56.1 (± 6.7) and 57.7 (± 4.1), respectively. All of the recruited women had not menstruated for at least 12 months.

9.2.2 Study design

The study protocol was approved by the Human Research Ethics Committee of Victoria University and consisted of a randomised, double-blind, placebo-controlled, parallel study involving a 2-week baseline period followed by a 12-week supplementation period. Each subject consumed two servings (200 mL each) of chilled (4°C) milk per day, before breakfast and before dinner. Subjects in the FS and NFS group ingested the same dosage of isoflavone at 80 mg per day (54 mg aglycone constituents) via 400 mL of soymilk. All batches of fermented soymilk contained a consistent population of BB12, at approximately 10^8 viable cells per mL. Fermented and non-fermented soymilks and casein-milk provided the same concentration of calcium at 112 mg per 200 mL serving. Each participant consumed a self-selected diet for the entire study (including baseline and supplementation periods) but was asked to exclude foods mentioned in section 7.2.2. A 7-day weighed food and beverage record (Appendix E) was completed during the baseline period and subjects were advised to follow this diet for each week of the milk supplementation period. From the recorded 7-day diet, daily energy, protein, carbohydrate, fat, dietary fibre and calcium intake were quantified for each subject using Food Works nutrition software (version 3.01) utilising a database of Australian foods (Xyris Software). Anthropometric measurements of height (m) and

bodyweight (kg) were recorded and the BMI (kg/m^2) of each subject calculated before and after the 12-week supplementation period.

9.2.3 Soymilk and casein-milk ingredients

Pure culture of BB12 was obtained from the culture collection mentioned in section 3.2.1 and stored under the conditions described in section 3.2.1. SPI159 and SG were obtained from the suppliers mentioned in section 6.2.1 and their macronutrient composition is also described in section 6.2.1. The total concentration of isoflavone in SPI159 and SG was 1.4 and 19.4 mg per gram, respectively (Table 6.1). SPI159 and SG contained 200 and 110 mg calcium per 100 g, respectively. Calcium caseinate or Alanate™ 391 (CC391) was obtained from New Zealand Milk Products (Wellington, New Zealand) and comprised ≥90 g of protein, <1 g of fat, and 1400 mg of calcium per 100 g. Vanilla flavour (in propylene glycol) was obtained from the supplier mentioned in section 7.2.4. Tri-calcium phosphate used for the fortification of fermented and non-fermented soymilk was obtained from Oppenheimer Pty. Ltd. (Silverwater, NSW, Australia).

9.2.4 Manufacture of soymilk

Soymilk was manufactured by reconstituting SPI159 and SG at a ratio of 8:2 (total of 4% w/v soy ingredients) so as to provide 40 mg isoflavone (27 mg aglycone constituents) per 200 mL serving; calculated based upon the concentration of isoflavone in SPI159 and SG (Table 6.1). For each subject of the FS and NFS group, fresh soymilk was prepared every fortnight for the duration of the study, with 28 bottles (equal to one carton) of refrigerated soymilk (200 mL each) delivered to each subject on six occasions. The volume of soymilk produced each fortnight depended on the current number of subjects participating in the study. For the manufacture of seven cartons of non-fermented soymilk, 1276 g of SPI159 and 324 g of SG were reconstituted in 40 L of filtered water tempered to 40°C, followed by heating and stirring at 70°C for 30 min during which 160 mL of vanilla flavour and 50 g of tri-calcium phosphate was added. The soymilk was then dispensed into eight bottles in 5 L quantities and autoclaved at 121°C for 15 min. After sterilisation, the bottles were cooled to approximately 75±5°C and 200 mL aliquots were dispensed into 200 sterile 200-mL

HDPE bottles (Cospak) under aseptic conditions using a 100 mL Trubor® bottle top dispenser (U-Lab). One-hundred millilitre aliquots from four randomly selected bottles of non-fermented soymilk were obtained under aseptic conditions and freeze dried as described in section 3.2.5 for the extraction of isoflavone and analysis using HPLC. The remaining 196 bottles of non-fermented soymilk were equally packaged into seven cartons and stored at 4°C prior to distribution. Forty litres of fermented soymilk was prepared in the same manner, but was cooled to 40°C after autoclaving (121°C/15 min) and inoculated with active culture of BB12 (see section 9.2.6).

9.2.5 Bacterial growth media

Rehydrated MRS agar (de Mann *et al.* 1960) containing additional 1% (w/v) D-glucose and 0.05% (w/v) L-cysteine·HCl was prepared as described in section 3.2.2. For the activation of BB12, 92 g of SPI159 and 23 g of food-grade glucose powder (Prahran Health Foods) per 2300 mL of filtered drinking water was reconstituted as described in section 3.2.3. Soymilk was then dispensed into glass bottles in 20, 50 and 500 mL quantities and sterilised by autoclaving at 121°C for 15 min.

9.2.6 Fermentation of soymilk by bifidobacteria

BB12 was activated by 3 successive transfers under the conditions described in section 7.2.7. Forty litres of sterile soymilk (prepared according to the steps described in section 9.2.4) was inoculated with active culture of BB12 (5% v/v). Two hundred millilitres of inoculated soymilk was dispensed into 200 sterile 200-mL HDPE bottles under aseptic conditions using a 100 mL Trubor® bottle top dispenser and incubated at 37°C for 24 h. Four bottles were randomly taken from the batch at 24 h of incubation and sample of 100 mL was withdrawn aseptically from each bottle for enumeration of viable BB12 populations (carried out in duplicate using the steps described in section 3.2.6) and the remainder stored at -20°C. Frozen samples were freeze dried as described in section 3.2.5 for the extraction of isoflavone and analysis using HPLC. The remaining 196 bottles of fermented soymilk were equally packaged into seven cartons and stored at 4°C prior to distribution.

9.2.7 Manufacture of casein-milk

Casein-milk was manufactured by reconstituting 4% (w/v) CC391. Like the production of soymilk, fresh casein-milk was prepared every fortnight for the duration of milk supplementation, with 28 bottles (equal to one carton) of refrigerated casein-milk (200 mL each) delivered to each subject of the CAS group on six occasions. The volume of casein milk produced each fortnight depended on the current number of subjects participating in the study. For the manufacture of seven cartons of casein-milk, 1600 g of CC391 was reconstituted in 40 L of filtered water tempered to 20±2°C, followed by heating and stirring at 70°C for 30 min during which 160 mL of vanilla flavour was added. The casein-milk was then dispensed into eight bottles in 5 L quantities and autoclaved at 121°C for 15 min. After sterilisation, the bottles were cooled to approximately 75±5°C and 200 mL was dispensed into 196 sterile 200-mL HDPE bottles under aseptic conditions using a 100 mL Trubor® bottle top dispenser. These bottles of sterile casein-milk were then equally packaged into seven cartons and stored at 4°C prior to distribution.

9.2.8 Isoflavone standards

Isoflavone standards of genistein, genistin, daidzein, daidzin, glycitein and glycitin were obtained from suppliers mentioned in section 7.2.8. Flavone, used as an ISTD, was obtained from Sigma. Mixed and single standards used for the analysis of isoflavones in soymilk were prepared as described in section 3.2.8.

9.2.9 Reversed-phase HPLC apparatus and reagents

Chromatographic analysis of isoflavones in soymilk were carried out on a Varian® 9000 series high performance liquid chromatograph (instrument components mentioned in section 7.2.9) connected to an Exsil™ (SGE International) C18-ODS (250 x 4.6 mm internal diameter; 5 μm) reversed-phase column. HPLC grade methanol and acetonitrile were purchased from Merck, and trifluoro-acetic acid, glacial acetic acid, sodium acetate and ammonium acetate from Sigma. All reagents used for the extraction of isoflavones and HPLC analyses were filtered through a 0.5 μm membrane (Millipore®) and mobile phases were degassed using nitrogen.

9.2.10 Extraction and HPLC analysis of isoflavones in soymilk

Randomly selected bottles from each batch of non-fermented and fermented soymilk were analysed for their isoflavone composition on a fortnightly basis to ensure that the concentration of isoflavone isomers consumed by each participant were consistent throughout the entire soymilk supplementation period. The extraction of isoflavone isomers (malonyl-, acetyl-, β-glucoside and aglycone forms) from 1.00 g of freeze-dried soymilk (non-fermented or fermented) was performed in duplicate using the method described by Setchell *et al.* (2001). HPLC gradient elution was used to separate isoflavone isomers and the conditions are described in section 7.2.10. The photodiode array UV/VIS detector was set at 259 nm and the approximate retention time (min) of each isoflavone isomer is mentioned in section 7.2.10. Quantification of isoflavone isomers in soymilk using multi-level calibration and an ISTD was like that described in section 3.2.10. Isoflavone concentrations were calculated back to wet basis and expressed as μmol per 200 mL of soymilk.

9.2.11 Collection and handling of blood and urine specimens

Each participant provided a blood and spot urine specimen at baseline and at week 12 of milk supplementation. Specimens were collected after an overnight fast (from 10 pm the night before the appointment). Dorevitch Pathology Clinical Trials Centre (Heidelberg, Victoria, Australia) prepared each biological specimen for frozen storage. From the blood specimens collected (25.5 mL drawn by venipuncture), serum was extracted (by clotting the blood for 30 min and centrifuging for 10 min at 3000 x g) and stored at -20°C for the testing of lipid profiles (total cholesterol, HDL-cholesterol, calculated LDL-cholesterol, and triglyceride), Lp(a), FSH, LH, and SHBG. The spot urine specimen consisted of the second urination of the morning and was stored at -70°C until the batch testing of bone resorption marker deoxypyridinoline (DPD).

9.2.12 Analysis of human biological fluids

Dorevitch Pathology Clinical Trials Centre analysed each serum specimen using automated biochemistry analysers. For each biomarker, serum specimens collected at baseline and at the endpoint from 36 subjects (total of 72 specimens) were analysed as a batch. Total cholesterol,

triglyceride, HDL-cholesterol and LDL-cholesterol were analysed using a Hitachi 917® (Boehringer Mannheim Corporation, Indianapolis, USA) and expressed on a mmol per litre basis. Analysis of Lp(a) was carried out on a Beckman IMMAGE® Immunochemistry system (Beckman Coulter Inc., Fullerton, CA, USA) and expressed on a mg per litre basis. The endocrine markers, FSH and LH, were analysed using a Bayer ADVIA Centaur® Immunoassay system (Bayer Diagnostics, Tarrytown, NY, USA), and SHBG on a DPC Immulite® 2000 Immunology analyser (Diagnostic Products Corporation, Los Angeles, CA, USA). FSH and LH were expressed on an IU per litre basis, whereas SHBG was expressed on a nmol per litre basis. For the analysis of DPD, a total of 72 urine specimens collected at the baseline and at the endpoint were analysed as a batch by Gribbles Pathology (Adelaide, SA, Australia) using a DPC Immulite® Classic Immunology analyser (Diagnostic Products Corporation); expressing DPD on a nmol per litre basis. In addition, each urine specimen was analysed for creatinine (CRE) using a modified version of the Jaffe method carried out on a modular-P unit (Roche Diagnostics Pty. Ltd., Castle Hill, NSW, Australia), with CRE expressed on a mmol per litre basis. The ratio of DPD (nmol) to CRE (mmol) was used to represent the urinary excretion of DPD as nmol per mmol of CRE.

9.2.13 Statistical analysis

Daily energy, macronutrient, calcium and cholesterol intake is presented as a mean ± standard deviation of 91 determinations for the FS and NFS group (13 subjects each recording their habitual diet for 7 days), and a mean ± standard deviation of 70 determinations for the CAS group (10 subjects recording their diet for 7 days). Urinary excretion of DPD and concentrations of endocrine and cardiovascular disease risk markers at baseline and after 12 weeks of supplementation for the FS and NFS group are presented as a mean ± standard deviation of 13 determinations. For the CAS group, the same markers at baseline and endpoint are presented as a mean ± standard deviation of 10 determinations. To find significant differences in bodyweight, BMI, nutrient intake, and the percentage change in biomarkers (from baseline to endpoint) between the FS, NFS and CAS group, means were analysed with one-way ANOVA and 95% confidence intervals using Microsoft® Excel Stat Pro™ as described by Albright *et al.* (1999). In addition, ANOVA and 95% confidence

intervals were also used to find significant differences between the baseline and endpoint means of each biomarker. ANOVA data with a $P<0.05$ was classified as statistically significant (two-sided test).

9.3 RESULTS AND DISCUSSION

9.3.1 BMI and bodyweight

The mean (± standard deviation) BMI (bodyweight) of the FS (n=13), NFS (n=13) and CAS (n=10) group at baseline was 24.9 ± 6.0 kg/m^2 (63.4 ± 13.7 kg), 26.7 ± 3.3 kg/m^2 (69.7 ± 8.4 kg), and 29.1 ± 7.6 kg/m^2 (76.4 ± 22.3 kg), respectively, with no significant differences in either BMI (P=0.24) or bodyweight (P=0.14) between the three groups. After 12 weeks of milk supplementation, there was no significant change in mean bodyweight from baseline measurements for the FS (P>0.9), NFS (P=0.88) and CAS (P>0.9) group. Hence, the mean BMI of the FS, NFS and CAS group at the conclusion of the study was 24.8 ± 5.7 kg/m^2, 26.9 ± 3.2 kg/m^2, and 29.2 ± 7.9 kg/m^2, respectively, similar to their respective baseline values (P>0.9).

9.3.2 Intake of dietary nutrients, viable bifidobacteria and isoflavones

Prior to commencing the 12 weeks of milk supplementation, each subject recorded their habitual diet for 7-days (excluding foods listed in section 7.2.2) and then closely followed this dietary intake for each week of the supplementation period. The mean (± standard deviation) daily energy intake for women in the FS, NFS and CAS group was 5965.4 ± 1522.0, 7968.5 ± 1815.8 and 7323.5 ± 2227.4 KJ per day, respectively, with no significant differences in daily energy intake between the three groups (P=0.09). Mean daily intake of macronutrients (expressed as grams per day) for the FS, NFS and CAS group are shown in Figure 9.1. The NFS and CAS group had a greater average daily intake of macronutrients compared to the FS group, but with no significant differences in the daily intake of carbohydrate, total fat, polyunsaturated fat, monounsaturated fat, saturated fat, and fibre (P>0.05). However, the protein intake of the NFS group was significantly greater than the FS group (P=0.02) (Figure 9.1). Furthermore, the habitual diet of the NFS group comprised a significantly greater mean daily intake of cholesterol than the FS group (P=0.02), at 283.5 ± 102.4

and 193.9 ± 41.0 mg per day, respectively. The mean daily cholesterol intake of the CAS group was between that of the NFS and FS groups at 214.5 ± 71.0 mg per day. For each group, approximately 19% of energy was in the form of protein, 47% in the form of carbohydrates and 32% as fat (total intake of polyunsaturated, monounsaturated and saturated fats) per day.

Due to the importance of calcium in bone health and its possible effects on bone resorption, the mean (± standard deviation) daily intake of calcium was also quantified in each supplementation group. The NFS and CAS group had similar mean daily intakes of calcium that were above the recommended dietary allowance of 800 mg per day, at 843 ± 142 and 866 ± 520 mg per day, respectively, and were both greater than the mean daily intake of calcium in the FS group at 556 ± 231 mg per day (P=0.12). In addition to the calcium obtained from their habitual diet, each subject from the FS, NFS and CAS group ingested 224 mg of calcium per day via 400 mL of soymilk and casein-milk. However, fermented soymilk, non-fermented soymilk and casein-milk did not provide equivalent levels of protein, carbohydrate, fat and fibre. According to the macronutrient composition of CC391 (section 9.2.3), casein-milk was a rich source of protein, providing approximately 14.4 g per day. The carbohydrate and fat content of casein-milk was negligible, considering it was prepared from a purified caseinate. In contrast, non-fermented soymilk made from a combination of SPI159 and SG provided 12.8 g of protein per day, similar to that of casein-milk, but also contained low levels of carbohydrate, fibre and fat, at 1.3, 0.10 and 1.0 g per daily serving of 400 mL, respectively. Fermented soymilk, on the other hand, may have contained a lower level of carbohydrate and protein than its non-fermented counterpart, as each of these nutrients could have been metabolised as a growth substrate during fermentation of bifidobacteria (Kamaly, 1997; Hou *et al.* 2000)

Viable populations of BB12 in each batch of fermented soymilk manufactured during the study and subsequently ingested by subjects in the FS group were in the range of 7.4 to 8.6 $\log_{10}$ CFU per mL, levels considered to be of therapeutic benefit (Gomes & Malcata, 1999; Shah, 2000). Furthermore, these populations of BB12 were similar to those in fermented soymilks examined in the isoflavone

bioavailability study reported in Chapter 7.0, which were in the range of 7.6 to 8.9 $\log_{10}$ CFU per mL. Populations of BB12 at >7.5 $\log_{10}$ CFU per mL appeared to reduce inter-individual variation in isoflavone excretion amongst women consuming fermented soymilk (that is, showing a high linearity in dose response), possibly by establishing a more consistent intestinal microflora amongst subjects in this group (Chapter 7.0). Hence, we ensured that each subject in the FS group was ingesting >7.5 $\log_{10}$ CFU per mL of viable BB12 via fermented soymilk to assess whether this potentially reduces the inter-individual variation in physiological response to isoflavone intake (in terms of biomarkers of disease risk) amongst a group of postmenopausal women.

The daily dosage of isoflavone implemented in this 12-week study was 80 mg per day via two servings of soymilk. Brouns (2002) stated that Asian people ingest between 20 and 100 mg isoflavone per day in their habitual diet with no adverse physiological effects reported in these populations. Hence, we chose an isoflavone dosage at the higher end of this concentration range. Considering the wide range of isoflavone doses employed in previous clinical studies on postmenopausal women (in the range of 30 to 135 mg per day) and the large inter-individual variability in physiological response to isoflavone intake reported earlier, it was difficult to find a specific dosage that was associated with a positive health effect. Some studies showed modest improvements in hormone levels (Duncan *et al.* 1999), lipid profiles (Gardner *et al.* 2001; Scheiber *et al.* 2001; Wangen *et al.* 2001) and bone turnover (Scheiber *et al.* 1999) in postmenopausal women ingesting 60 to 80 mg isoflavone per day for 12 to 13 weeks. However, in addition to providing a soymilk that would administer a dosage of isoflavone in the range of 60 to 80 mg per day, it would also need to be desirable to drink, bearing in mind that the women in the FS and NFS group were required to consume soymilk on a daily basis for 12 weeks. From our previous study (Chapter 7.0), the majority of women preferred the flavour and mouth-feel of non-fermented and fermented SPI/SG40, providing a 40 mg dose of isoflavone per 200 mL serving. Hence, the formulation of SPI/SG40 was implemented in this study and in this case two servings were ingested per day to reach the 80 mg dosage level. The isoflavone composition of non-fermented and fermented SPI/SG40 (shown in Table 7.1) was compared to each fresh batch of non-fermented and fermented soymilk produced in this study on a fortnightly basis to ensure that the concentration of

isoflavone isomers ingested per day for the duration of the study were as consistent as possible. More than 80% of the randomly selected bottles of fermented (after 24 h of incubation) and non-fermented soymilk manufactured during this study contained equivalent concentrations of isoflavone isomers found in fermented and non-fermented SPI/SG40 shown in Table 7.1 (that is, within the range of the mean ± standard deviation of each isomer). With respect to the total isoflavone concentration, non-fermented soymilk consumed in this study generally contained 9% in an unconjugated aglycone configuration, whereas fermented soymilk comprised a significantly greater proportion of free aglycone at approximately 57% of total isoflavone ($P<0.05$).

9.3.3 Influence of fermented and non-fermented soymilk on sex steroid hormones in postmenopausal women

Menopause is typically associated with elevated levels of FSH and LH, and is a consequence of diminishing levels of endogenous oestrogens resulting from ovarian dysfunction. Changes in SHBG concentration have a considerable influence on the amount of free and bound oestrogen, with only the free form able to bind to ER sites. Changes in total oestrogen concentration result in relatively small changes in the size of the free oestrogen fraction (Tham *et al.* 1998). Geola *et al.* (1980) reported that conjugated steroidal oestrogens ingested by postmenopausal women (n=21) at a dose of 0.15 mg per day caused significant increases in SHBG ($P<0.05$). Furthermore, SHBG was a more sensitive indicator of oestrogenic response than FSH and LH in postmenopausal women, with FSH and LH only showing significant decreases in their levels at oestrogen doses of 0.3 mg and 0.625 mg per day, respectively. Clinical studies carried out to examine the potential oestrogenic effects of isoflavones (ingested via non-fermented soy foods) on levels of SHBG, FSH and LH in postmenopausal women have not been as convincing as those studies administering conjugated oestrogens, even at doses between 80 and 165 mg isoflavone per day (Baird *et al.* 1995; Duncan *et al.* 1999; Pino *et al.* 2000; Persky *et al.* 2002). Such high doses have been used to compensate for the lower oestrogenic activity of isoflavone compared to conjugated oestrogens (Baird *et al.* 1995).

Mean concentrations of FSH, LH and SHBG in the FS, NFS and CAS group at baseline and after 12 weeks of supplementation are shown in Table 9.1, with the mean percentage change in

concentration of each of these hormones (from baseline to endpoint) shown in Figure 9.2. Supplementation of fermented and non-fermented soymilk caused a decrease in the levels of FSH and LH ($P>0.05$), with greater reductions of both hormones occurring in the NFS group rather than the group ingesting the isoflavone aglycone-enriched fermented soymilk containing viable BB12 (Table 9.1). FSH and LH decreased by 11.5 and 7.0 IU/L after 12 weeks of supplementation of non-fermented soymilk, respectively. Of the two hormones, LH appeared to be more responsive to soymilk intake, decreasing by 20.8% in the NFS group and 9.9% in the FS group (Figure 9.2). Supplementation with casein-milk did not reduce the concentrations of LH in postmenopausal women, with the CAS group actually showing an increase in LH of 3.0% after 12 weeks (Figure 9.2). However, a 7.8% reduction in FSH was observed in the group of women consuming casein-milk, which was greater than the 4.7% reduction in FSH in the FS group ($P=0.97$) (Figure 9.2). In an earlier study, Baird *et al.* (1995) reported that there were greater reductions in FSH than LH after 4 weeks of daily soy food ingestion providing 165 mg isoflavone per day ($P>0.05$), but with decreases in LH and FSH also observed in postmenopausal women consuming the control diet containing no soy foods ($P>0.05$). Duncan *et al.* (1999) also reported that soy isoflavone intake had a greater effect on FSH levels than LH after 93 days of treatment. In that study, postmenopausal women ingesting 7 and 65 mg isoflavone per day via soy protein showed small but significant reductions in FSH ($P=0.01$), accompanied by very little change in LH levels ($P>0.05$) (Duncan *et al.* 1999). Considering that there were modest oestrogenic effects observed in the group ingesting as little as 7 mg isoflavone per day, Duncan *et al.* (1999) proposed that reductions in FSH may be due to the combined effects of soy isoflavones and other components of soy. In our study, consumption of non-fermented soymilk appeared to be more effective than fermented soymilk in reducing levels of FSH and LH in postmenopausal women, and this may be due to differences in composition between fermented and non-fermented soymilk.

Due to the considerable inter-individual variation in baseline and endpoint values of FSH and LH for each group, represented by the large values of standard deviation, there were no significant differences in mean concentrations of FSH and LH before and after supplementation of fermented soymilk, non-fermented soymilk or casein-milk ($P>0.05$) (Table 9.1). Similarly, Teede *et al.* (2001)

found no significant differences in concentrations of FSH and LH in postmenopausal women before and after 3 months of either soy protein (118 mg isoflavone per day) or casein protein intake ($P>0.05$). In our study, there was also considerable inter-individual variation in physiological response to both fermented and non-fermented soymilk intake, represented by large values of standard deviation for the mean percentage change in FSH and LH (Figure 9.2). Consequently, there were no significant differences in mean percentage change in either FSH or LH between the FS, NFS and CAS group ($P>0.05$). Due to the lack of statistical significance, there was no strong evidence to indicate that the consumption of non-fermented soymilk was more effective than fermented soymilk and casein-milk in reducing concentrations of FSH and LH in postmenopausal women, despite the trend shown in Table 9.1 and Figure 9.2.

Moderate rises in SHBG were observed in the groups consuming the non-fermented and fermented soymilk (Table 9.1 and Figure 9.2). The mean increase in SHBG after 12 weeks of soymilk supplementation was very similar for the FS and NFS group, at 3.4 and 3.6 nmol/L, respectively, with no significant difference between baseline and endpoint means for either of the groups ($P>0.05$) (Table 9.1). As shown in Figure 9.2, this corresponded to an average increase in SHBG of 7.9% for the FS group and 5.7% for the NFS group. In contrast, the CAS group showed a decrease in SHBG after 12 weeks of casein-milk supplementation, with a mean reduction of 4.0%. Despite the trend toward an increase in SHBG resulting from soymilk supplementation and the decrease in SHBG observed in women consuming the placebo, there were no significant differences in mean percentage change between the FS, NFS and CAS group ($P=0.22$) (Figure 9.2). Earlier studies by Duncan *et al.* (1999) and Pino *et al.* (2000) also found that the intake of soy isoflavones increased concentrations of SHBG in postmenopausal women, but both these investigations reported significant rises in SHBG as a result of soy food consumption ($P<0.05$). Pino *et al.* (2000) reported that isoflavone intake (via soymilk) had a pronounced effect on SHBG in postmenopausal women whose baseline values of SHBG were at the low end of the concentration range. When examining the baseline and endpoint values of SHBG for each woman in the FS and NFS group, we found no association between low baseline values of SHBG and greater increases in SHBG after soymilk supplementation.

As a group, women ingesting fermented and non-fermented soymilk did not show significantly different changes in SHBG, FSH and LH compared to the placebo group to suggest that soy intervention had a positive effect on hormone profiles (Table 9.1 and Figure 9.2). Furthermore, the FS group ingesting a higher proportion of isoflavone aglycone and viable BB12 via fermented soymilk showed no greater oestrogenic response, in terms of adjustments in SHBG, FSH and LH, than the group consuming non-fermented soymilk. However, when baseline and endpoint values of SHBG, FSH and LH were examined in each subject of the FS, NFS and CAS group, decreases in both FSH and LH and an increase in SHBG was observed in 5 of the 13 subjects in the FS group. In the NFS and CAS group, only 3 and 2 women, respectively, showed these adjustments in all three hormones. Hence, from individual data the consumption of fermented soymilk appeared to have a greater oestrogenic effect than non-fermented soymilk.

9.3.4 Lipids and Lp(a) in postmenopausal women ingesting fermented soymilk, non-fermented soymilk and casein-milk

Considering that HRT has shown to lower the risk of cardiovascular disease in postmenopausal women (Arjmandi, 2001), the weak oestrogenic activity of isoflavones may also have a similar effect on cardiovascular disease risk. The majority of clinical trials investigating the efficacy of soy isoflavones in the prevention of cardiovascular disease in postmenopausal women have examined changes in lipid profiles during treatment and reported highly variable results (Baum *et al.* 1998; Washburn *et al.* 1999; Gardner *et al.* 2001; Scheiber *et al.* 2001; Wangen *et al.* 2001; Jenkins *et al.* 2002). Lp(a), the cholesterol-carrying particle in the blood which is structurally similar to LDL-cholesterol but not responsive to many conventional approaches (changes in diet and ingestion of pharmaceutical products) used to lower LDL-cholesterol, is an independent risk factor for cardiovascular disease that has been shown to decrease as a result of HRT (Kim *et al.* 1994; Shewmon *et al.* 1994). Previous studies by Hodgson *et al.* (1998), Teede *et al.* (2001), Wangen *et al.* (2001) and Tonstad *et al.* (2002) examined whether the oestrogenic nature of isoflavones had a similar Lp(a)-reducing effect as HRT, but found very little evidence to support their hypothesis. In a novel approach to enhance the potential effects of soy food on blood lipid profiles and Lp(a), we

administered isoflavones and bifidobacteria, both of which are associated with potential cholesterol-lowering properties, to postmenopausal women via a fermented soymilk.

Baseline and endpoint concentrations of total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol and Lp(a) in postmenopausal women consuming either fermented soymilk, non-fermented soymilk or casein-milk for 12 weeks are shown in Table 9.2. The mean percentage from baseline to endpoint in each of these lipids and Lp(a) for the FS, NFS and CAS group are shown in Figure 9.3. There were no significant differences between baseline and endpoint means of total cholesterol, triglyceride and LDL-cholesterol to indicate that the consumption of fermented soymilk or non-fermented soymilk had a cholesterol-lowering effect in postmenopausal women ($P>0.05$) (Table 9.2). Likewise, women consuming the placebo of casein-milk did not show significant alterations in their lipid profiles ($P>0.05$) (Table 9.2). However, there was a trend toward a positive correlation between fermented soymilk consumption and HDL-cholesterol concentrations in postmenopausal women, with a mean increase of 0.23 mmol/L ($P=0.08$) (Table 9.2). This was supported by a significant difference in mean percentage change in HDL-cholesterol between the FS and CAS group ($P=0.01$) (Figure 9.3). Women in the FS group showed a 19.9% increase in HDL-cholesterol after 12 weeks of fermented soymilk supplementation, whereas the CAS group showed a 2.9% decrease in HDL-cholesterol. Even though the mean increase in HDL-cholesterol in the FS group was 2.4 times greater than that occurring in the NFS group (at 8.2%), there was no significant difference in mean percentage of HDL-cholesterol observed between these two groups to indicate that the ingestion of fermented soymilk had a greater positive effect on HDL-cholesterol than its non-fermented equivalent ($P>0.05$). Similarly, Scheiber *et al.* (2001) reported that 12 weeks of soy food consumption providing 60 mg isoflavone per day significantly increased mean levels of HDL-cholesterol in a group of postmenopausal women. The mean increase in HDL-cholesterol was only 3.7% in the study by Scheiber *et al.* (2001), considerably lower than the increase in HDL-cholesterol observed in the FS group. However, Scheiber *et al.* (2001) also reported a decrease in total cholesterol after 12 weeks of soy food consumption. For the FS and NFS group, mean percentage changes (from baseline to endpoint) in total cholesterol, triglyceride and LDL-cholesterol actually showed a trend toward an increase in each of these lipids rather than a decrease.

Only the consumption of casein-milk showed a reduction in total cholesterol (7.2%) and LDL-cholesterol (11.5%) after 12 weeks of supplementation, but due to the considerable inter-individual variation within each group there were no significant differences found between the reductions in total cholesterol and LDL-cholesterol shown in the CAS group and the increases in these lipids observed in the FS and NFS group ($P>0.05$).

Figure 9.4 shows the mean ratio of total cholesterol to HDL-cholesterol and the mean ratio of LDL-cholesterol to HDL-cholesterol at baseline and at the end of the study for each of the supplementation groups. There was a trend toward a reduction in the ratio of total cholesterol to HDL-cholesterol and LDL-cholesterol to HDL-cholesterol in women consuming fermented and non-fermented soymilk, but this was also observed in women consuming the casein-milk. Furthermore, there were no significant differences between baseline and endpoint ratios for either of the groups ($P>0.05$) (Figure 9.4). In contrast, Jenkins *et al.* (2002) reported that postmenopausal women ingesting 10 and 73 mg isoflavone per day showed a significant reduction in the ratio of total to HDL-cholesterol and LDL-cholesterol to HDL-cholesterol compared to the placebo group (diet of dairy foods) after only 1 month of treatment ($P<0.05$). In our study, even though the FS group showed a large percentage increase in HDL-cholesterol (Figure 9.3), there was no significant improvement in HDL-cholesterol ratios (Figure 9.4) because of the increases in total cholesterol and LDL-cholesterol which also occurred in the FS group (Figure 9.3).

At baseline and at the conclusion of the study, there was large inter-individual variation in Lp(a) levels observed in each of the study groups (Table 9.2). There were only slight reductions in Lp(a) levels after 12 weeks of fermented soymilk and non-fermented soymilk consumption, with no significant difference between baseline and endpoint means for the FS and NFS group ($P>0.05$) (Table 9.2). When expressed on a percentage change basis (Figure 9.3), this corresponded to an 8.2% reduction in Lp(a) in the FS group, greater than the 2.7% and 0.3% reduction shown by the NFS and CAS group, respectively, but with no significant differences between the groups ($P>0.05$). Nevertheless, the trend toward a reduction in Lp(a) amongst women consuming fermented soymilk was in contrast to results reported by Teede *et al.* (2001) and Tonstad *et al.* (2002) who showed that

soy protein intake increased levels of Lp(a) in postmenopausal women. Similar to our results, Hodgson *et al.* (1998) also observed a reduction in Lp(a) levels in postmenopausal women after isoflavone treatment, in the magnitude of 5 to 6%, and like our findings there was no significant difference between baseline and endpoint means of Lp(a) ($P>0.05$).

9.3.5 Effects of fermented soymilk on bone resorption

Diminishing oestrogen levels in women resulting from ovarian dysfunction and menopause are associated with elevated bone resorption caused by a rise in osteoclastic activity. Consequently, postmenopausal women are more susceptible to osteoporosis. Despite its proposed adverse cancer-related effects, HRT effectively lowers the risk of osteoporosis in postmenopausal women (Arjmandi, 2001). The bone-sparing effects of isoflavones in postmenopausal women have also been examined in epidemiological and clinical studies, due to their oestrogenic nature, to assess whether they are an effective and safe alternative to HRT (Potter *et al.* 1998; Scheiber *et al.* 1999; Alekel *et al.* 2000; Mei *et al.* 2001; Lydeking-Olsen *et al.* 2002; Uesugi *et al.* 2002; Chen *et al.* 2003). Markers of bone formation and resorption have been commonly used to investigate the effects of soy isoflavone ingestion on osteoblastic and osteoclastic activity in postmenopausal women, respectively, and their influence on bone health and condition. In a study by Scheiber *et al.* (1999), 50 postmenopausal women consuming a dosage of 60 to 70 mg isoflavone per day (via soymilk) for 12 weeks, showed a reduction in the rate of bone loss with a significant reduction in the bone resorption marker urinary N-telopeptide and an increase in bone formation marker osteocalcin ($P<0.05$). Uesugi *et al.* (2002) examined bone turnover in Japanese women ingesting 61.8 mg per day and found significant reductions in urinary excretion of DPD after only 4 weeks of treatment ($P<0.05$), but no apparent effects on bone formation marker osteocalcin ($P>0.05$). In a longer term study, Alekel *et al.* (2000) reported that perimenopausal women ingesting 80.4 mg isoflavone per day (via soy protein) for 24 weeks showed very little change in markers of bone formation and resorption, despite the bone-sparing effect observed in the lumbar spine region of subjects ingesting isoflavone. On the whole, these earlier clinical studies administered a glucoside-rich form of isoflavone and have reported highly variable results.

The mean urinary excretion of bone resorption marker DPD before and after supplementation of fermented soymilk, non-fermented soymilk or casein-milk by postmenopausal women is shown in Table 9.3, and the mean percentage change in urinary DPD (from baseline to endpoint) is shown in Figure 9.5. After 12 weeks of supplementation, only the ingestion of fermented soymilk showed a trend toward a reduction in bone resorption, with urinary DPD decreasing by 2.4 nmol/mmol of CRE, but no significant difference was evident between baseline and endpoint means of the FS group (P=0.16) (Table 9.3). In the NFS and CAS group, there was very little difference between baseline and endpoint concentrations of urinary DPD (P>0.05) (Table 9.3). However, expressing changes in urinary DPD (from baseline to endpoint) on a percentage basis highlighted considerable differences between the supplementation groups (Figure 9.5). In contrast to the increase in DPD excretion shown by women in the NFS and CAS group (10.3% and 3.3%, respectively), women consuming fermented soymilk showed a 17.3% reduction in the urinary excretion of DPD (P=0.05). Despite the lack of statistical evidence due to the large inter-individual variation observed in each group, exemplified by the values of standard deviation shown in Figure 9.5, there is a clear trend showing a potential for fermented soymilk to reduce bone resorption in postmenopausal women, and it being more effective than a non-fermented soymilk. The enhanced bone-sparing actions of fermented soymilk may have been due to its greater proportion of isoflavone in an aglycone configuration compared to its non-fermented counterpart. Taking into consideration the influence of calcium on bone maintenance, women in the FS group had a lower mean daily intake of calcium than the NFS and CAS group (section 9.3.2), supporting the role of fermented soymilk in preventing bone resorption.

9.4 CONCLUSIONS

When compared to the non-fermented and placebo groups, there was very little evidence to indicate that ingesting fermented soymilk providing a dosage of 80 mg isoflavone per day had a greater oestrogenic effect on LH, FSH and SHBG in postmenopausal women. However, on an individual basis, 5 of the 13 subjects in the fermented soymilk group did show a reduction in both LH and FSH accompanied by an increase in SHBG after 12 weeks, more than the number of women showing similar hormonal changes in response to non-fermented soymilk and casein-milk. With respect to

cardiovascular disease risk, women consuming fermented soymilk did show a significant increase in HDL-cholesterol levels compared to the casein-milk group, but were not accompanied by reductions in total cholesterol, LDL-cholesterol and triglyceride. Additionally, there was a trend toward a reduction in Lp(a) in women consuming fermented soymilk, except that it was not significantly different to the group consuming non-fermented soymilk or the placebo. Thus, stronger evidence is required to suggest that the concomitant ingestion of isoflavone and bifidobacteria may protect postmenopausal women against cardiovascular disease. Nevertheless, the group of women consuming fermented soymilk did show a large reduction in the urinary excretion of bone resorption marker DPD compared to the non-fermented soymilk and placebo groups. This result indicated that isoflavone aglycone-enriched fermented soymilk may be more effective than non-fermented soymilk in protecting against bone loss in postmenopausal women. Overall, there was a lack of clear evidence to indicate that fermented soymilk improves hormone profiles and reduces the risk of cardiovascular disease in postmenopausal women, but there was some evidence to suggest that it may prevent osteoporosis.

Table 9.1 Concentrations of FSH, LH and SHBG in serum specimens collected from three groups of postmenopausal women before and after 12 weeks of supplementation[1] of either fermented soymilk, non-fermented soymilk or a placebo of casein-milk (mean ± standard deviation)

Group	**FSH**	**LH**	**SHBG**
Time interval	**(IU/L)**	**(IU/L)**	**(nmol/L)**
FS (n=13)			
Baseline	74.4±35.2	37.2±11.0	50.5±16.6
Endpoint	66.8±35.0	33.5±10.8	53.9±18.7
P-value[2]	0.59	0.40	0.62
NFS (n=13)			
Baseline	64.3±26.8	34.0±7.2	49.9±23.5
Endpoint	52.8±22.4	27.0±12.8	53.5±28.8
P-value[2]	0.26	0.11	0.74
CAS (n=10)			
Baseline	63.0±25.1	24.7±10.2	43.3±24.6
Endpoint	55.7±22.0	23.3±6.8	42.1±25.7
P-value[2]	0.50	0.72	0.92

FSH: Follicle-stimulating hormone; LH: Luteinising hormone; SHBG: Sex hormone binding globulin; FS: Fermented soymilk providing 54 mg isoflavone aglycone constituents per day and viable populations of *B. animalis* BB12 (~8 $\log_{10}$ CFU per mL); NFS: Non-fermented soymilk providing 54 mg isoflavone aglycone constituents per day; CAS: Casein-milk.

[1]Two 200-mL servings of milk per day.

[2]Each pair of means (baseline versus endpoint) were analysed using one-way ANOVA.

Table 9.2 Concentrations of total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol and Lp(a) in serum specimens collected from three groups of postmenopausal women before and after 12 weeks of supplementation[1] of either fermented soymilk, non-fermented soymilk or a placebo of casein-milk (mean ± standard deviation)

Group Time interval	Total cholesterol (mmol/L)	Triglyceride (mmol/L)	HDL-cholesterol (mmol/L)	LDL-cholesterol (mmol/L)	Lp(a) (mg/L)
FS (n=13)					
Baseline	5.39±0.89	1.10±0.73	1.39±0.37	3.48±0.69	343.7±465.4
Endpoint	5.79±0.58	1.13±0.43	1.62±0.27	3.65±0.54	339.9±475.6
P-value[2]	0.19	0.89	0.08	0.49	0.98
NFS (n=13)					
Baseline	4.99±0.94	1.08±0.49	1.38±0.21	3.11±0.72	154.6±128.3
Endpoint	5.05±1.04	1.10±0.48	1.48±0.28	3.07±0.70	149.9±133.2
P-value[2]	0.89	0.93	0.33	0.89	0.93
CAS (n=10)					
Baseline	5.60±1.10	1.39±1.19	1.56±0.47	3.39±0.85	394.8±369.4
Endpoint	5.13±1.24	1.19±0.63	1.52±0.54	2.90±0.77	374.7±345.0
P-value[2]	0.41	0.66	0.89	0.25	0.90

HDL: High-density lipoprotein; LDL: Low-density lipoprotein; Lp(a): Lipoprotein(a); FS: Fermented soymilk providing 54 mg isoflavone aglycone constituents per day and viable populations of *B. animalis* BB12 (~8 $\log_{10}$ CFU per mL); NFS: Non-fermented soymilk providing 54 mg isoflavone aglycone constituents per day; CAS: Casein-milk.

[1]Two 200-mL servings of milk per day.

[2]Each pair of means (baseline versus endpoint) were analysed using one-way ANOVA.

Table 9.3 Urinary excretion of bone resorption marker deoxypyridinoline by three groups of postmenopausal women before and after 12 weeks of supplementation[1] of either fermented soymilk, non-fermented soymilk or a placebo of casein-milk (mean ± standard deviation)

Group Time interval	Deoxypyridinoline (nmol/mmol of CRE)
FS (n=13)	
Baseline	11.7±4.2
Endpoint	9.3±2.4
P-value[2]	0.16
NFS (n=13)	
Baseline	8.9±1.9
Endpoint	9.6±2.1
P-value[2]	0.44
CAS (n=10)	
Baseline	10.4±2.6
Endpoint	10.3±2.6
P-value[2]	0.97

CRE: Urinary creatinine (mmol/L); FS: Fermented soymilk providing 54 mg isoflavone aglycone constituents per day and viable populations of *B. animalis* BB12 (~8 $\log_{10}$ CFU per mL); NFS: Non-fermented soymilk providing 54 mg isoflavone aglycone constituents per day; CAS: Casein-milk.

[1]Two 200-mL servings of milk per day.

[2]Each pair of means (baseline versus endpoint) were analysed using one-way ANOVA.

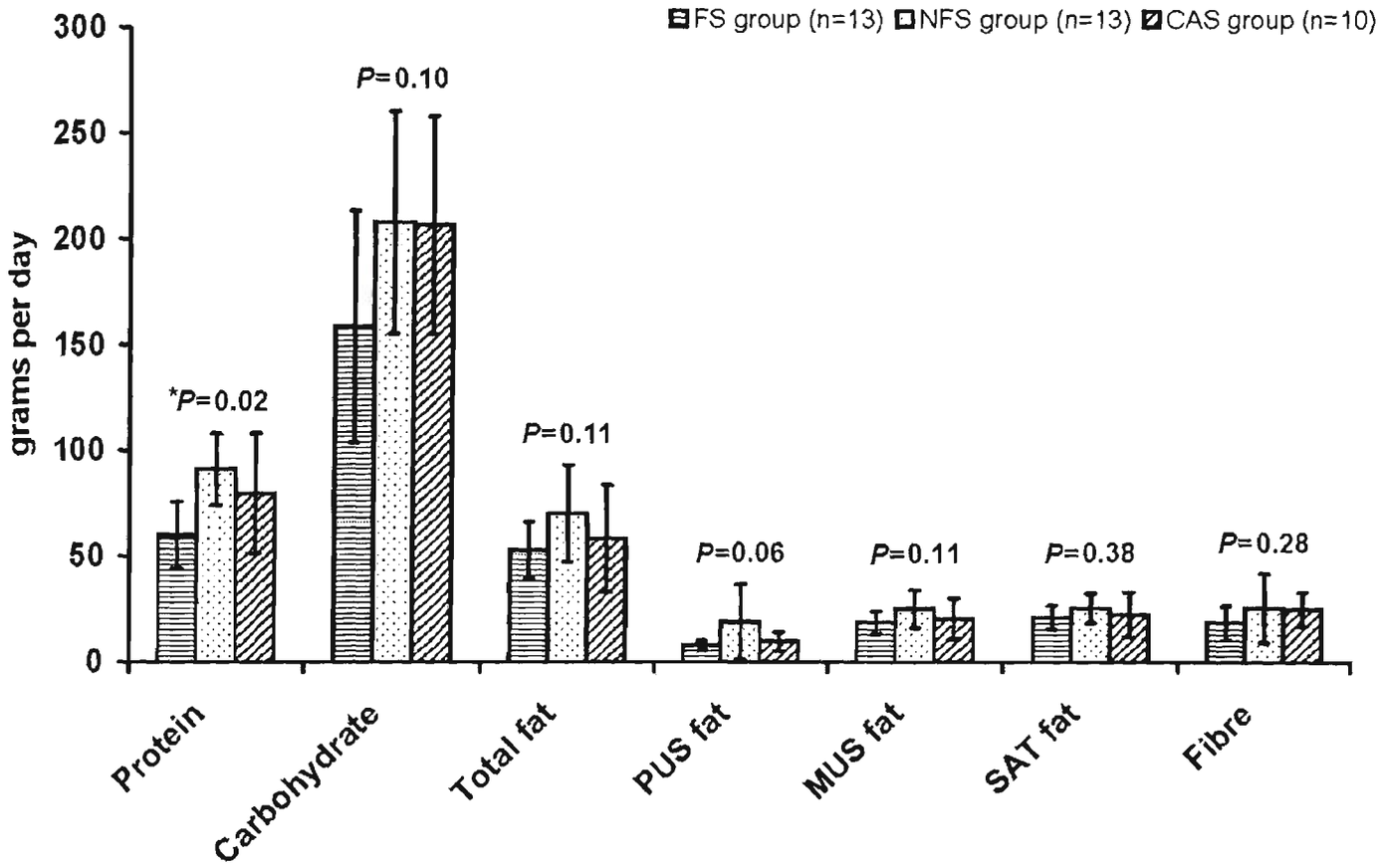

Figure 9.1 Daily intake of macronutrients (grams per day) in the fermented soymilk (FS), non-fermented soymilk (NFS), and casein-milk (CAS) group during the 7-day baseline diet. This self-selected diet was followed for each week of the milk supplementation period (total of 12 weeks). Columns and error bars for the FS, NFS and CAS group represent a mean ± standard deviation. Means of the FS, NFS and CAS group arranged as three columns were analysed using one-way ANOVA. *Significant difference in protein intake between the FS and NFS group. PUS: Polyunsaturated fats; MUS: Monounsaturated fats; SAT: Saturated fats.

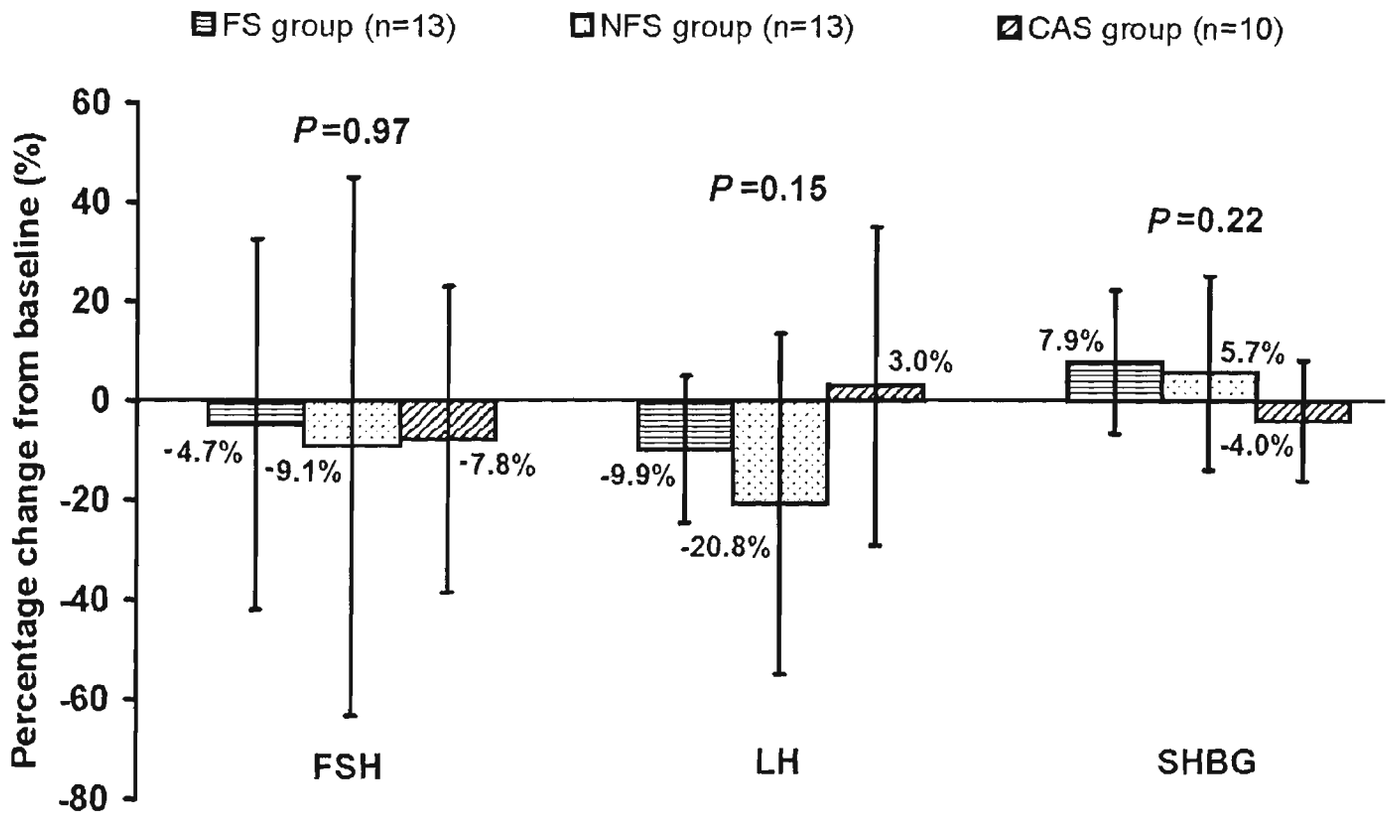

Figure 9.2 Mean percentage change (from baseline to endpoint) in follicle-stimulating hormone (FSH), luteinising hormone (LH), and sex hormone binding globulin (SHBG) in the fermented soymilk (FS), non-fermented soymilk (NFS), and casein-milk (CAS) group. Columns and error bars represent a mean ± standard deviation. Means of the FS, NFS and CAS group arranged as three columns were analysed using one-way ANOVA.

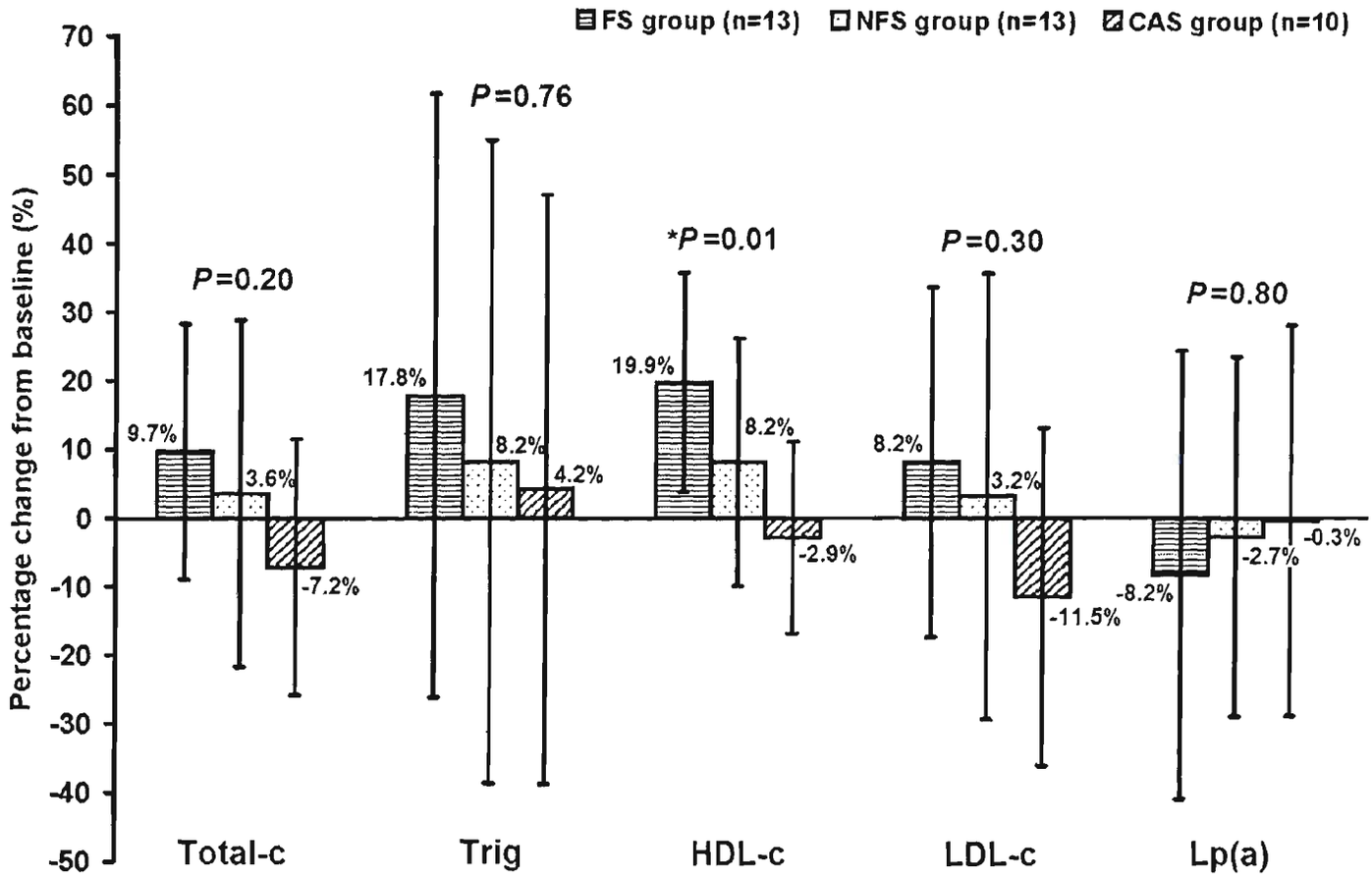

Figure 9.3 Mean percentage change (from baseline to endpoint) in total cholesterol (Total-c), triglyceride (Trig), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), and lipoprotein(a) [Lp(a)] in the fermented soymilk (FS), non-fermented soymilk (NFS), and casein-milk (CAS) group. Columns and error bars represent a mean ± standard deviation. Means of the FS, NFS and CAS group arranged as three columns were analysed using one-way ANOVA. *Significant difference between the FS and CAS group.

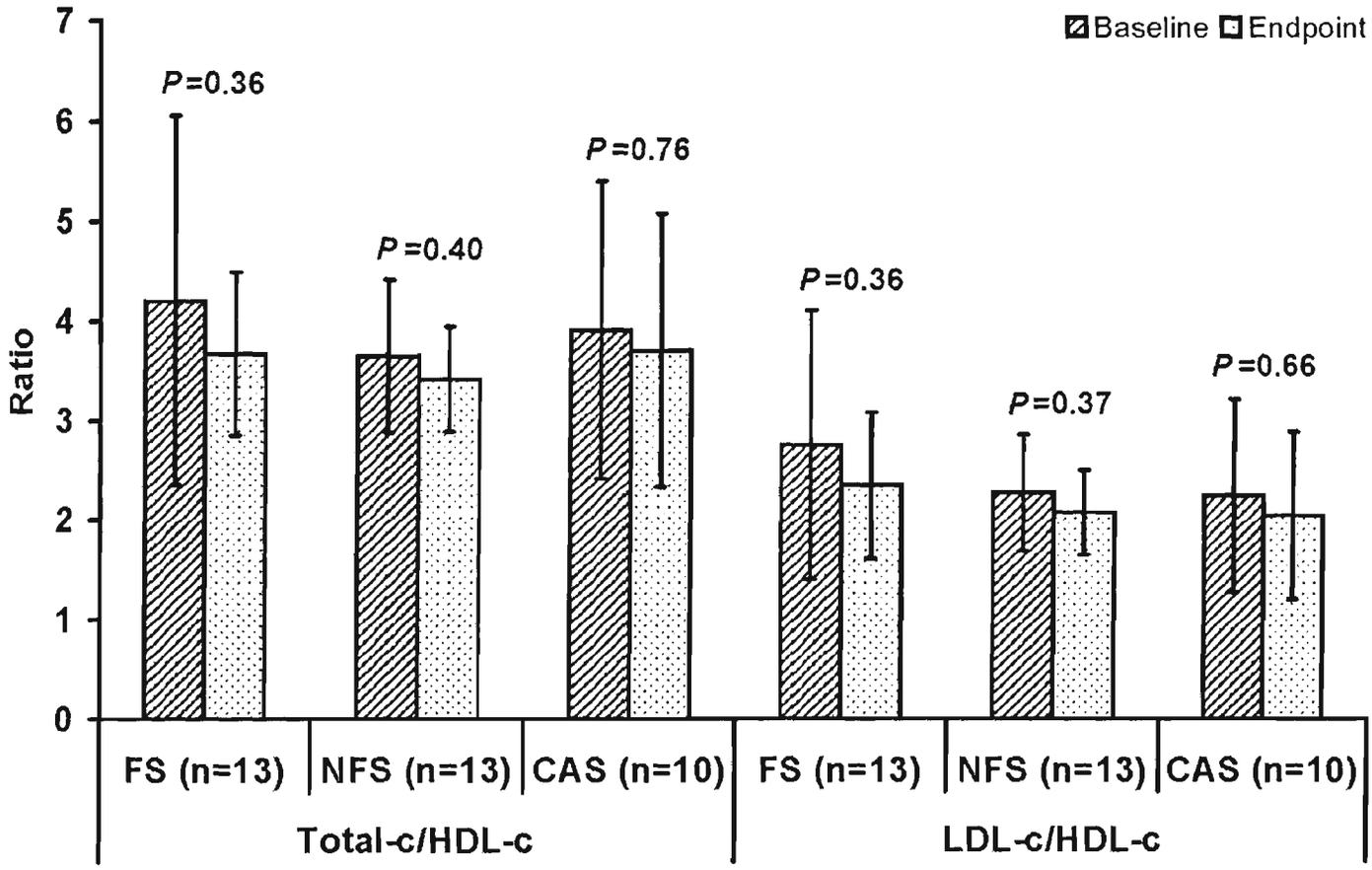

Figure 9.4 Mean ratio of total cholesterol (Total-c) to high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) to HDL-c before and after 12 weeks of supplementation in the fermented soymilk (FS), non-fermented soymilk (NFS), and casein-milk (CAS) group. Columns and error bars represent a mean ± standard deviation. Each pair of means (baseline versus endpoint) were analysed using one-way ANOVA.

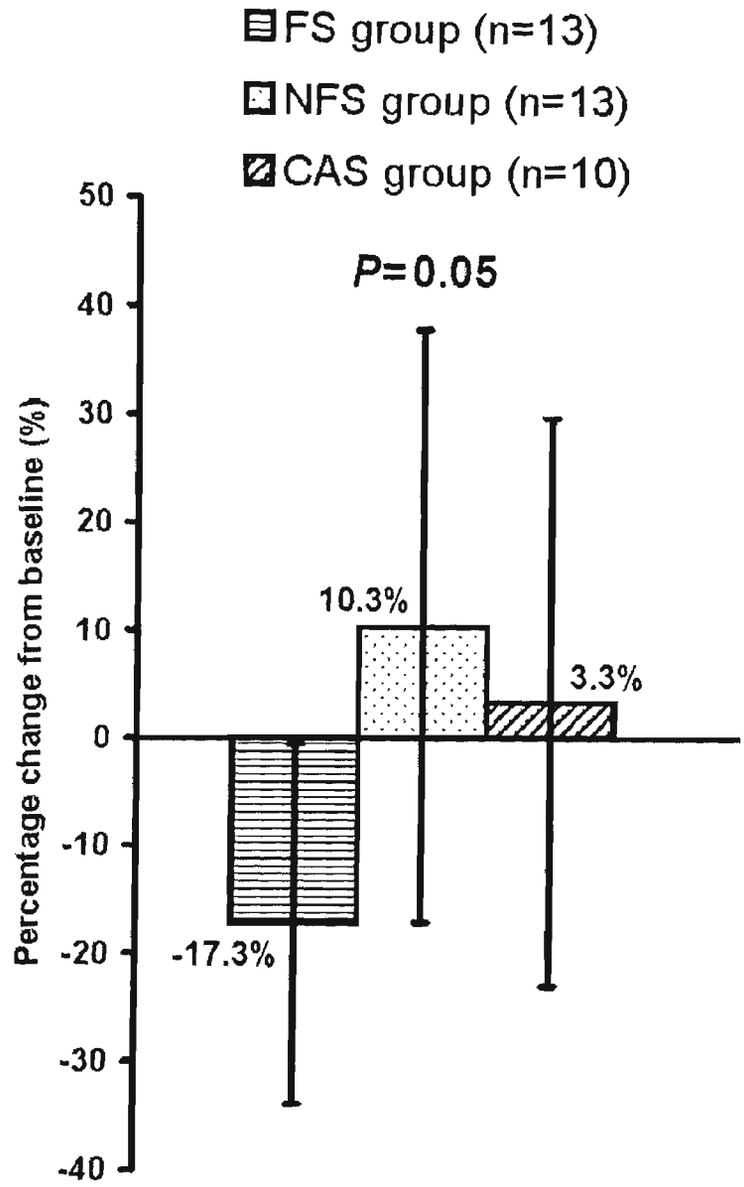

Figure 9.5 Mean percentage change (from baseline to endpoint) in urinary bone resorption marker deoxypyridinoline in the fermented soymilk (FS), non-fermented soymilk (NFS), and casein-milk (CAS) group. Columns and error bars represent a mean ± standard deviation. Means of the FS, NFS and CAS group were analysed using one-way ANOVA.

10.0 Overall Conclusions and Future Research Direction

This study showed that in addition to their well known probiotic effects, bifidobacteria may play an important role in improving the nutritional quality of soymilk made from SPI. We found that the metabolic activities of bifidobacteria effectively modified the composition of SPI-soymilk during fermentation, causing significant changes in some of the soy constituents of greatest interest to manufacturers of soy foods. The β-glucosidase activity of *Bifidobacterium* appeared to be involved in hydrolysing isoflavone glucosides in SPI-soymilk, subsequently releasing the aglycone form which is associated with oestrogen-like activity and is the structure absorbed in the intestinal tract. Interestingly, the extent of isoflavone biotransformation into bioactive aglycone forms was not improved by providing a more favourable growth environment for bifidobacteria. Instead, a greater proportion of isoflavone glucoside was metabolised in SPI-soymilk containing less fermentable sugars, possibly forcing bifidobacteria to scavenge for alternate carbon sources during growth. Bifidobacteria also metabolised the oligosaccharide (raffinose and stachyose) content of SPI-soymilk, the soluble sugars which are the cause of gas production and flatulence in some consumers. During fermentation, bifidobacteria utilised these oligosaccharides as a growth substrate. This was influenced by their capacity to produce α-galactosidase. Those strains of *Bifidobacterium* showing the greatest α-galactosidase activity metabolised the greatest concentration of oligosaccharides, exhibited the best growth in SPI-soymilk, and produced the greatest concentration of organic acid by-products (lactic and acetic acid). In addition, fermentation by bifidobacteria reduced the concentration of hexanal and pentanal in SPI-soymilk. These aldehyde compounds are associated with the objectionable odour and 'beany' flavour of soymilk, disliked by Western people accustomed to dairy milk.

Combining SPI with isoflavone-rich SG in the manufacture soymilk (SPI/SG-soymilk) was a simple and effective way of increasing the concentration of isoflavone to levels ingested by Asian populations and dosages considered to be of possible health benefit (60 to 80 mg per day). Fermenting SPI/SG-soymilk with bifidobacteria enriched the concentration of bioactive isoflavone

aglycone. In addition, viable populations of bifidobacteria attained during fermentation, which were in the range considered to be of therapeutic benefit (10^8 viable cells per mL), remained constant during refrigerated storage. However, some aspects of this work aimed toward developing an isoflavone aglycone-enriched, probiotic soymilk still require further research to refine this product and to attain a better understanding of its composition and possible health benefit to consumers. An important factor which affected the efficiency of manufacturing this product was the long fermentation time (that is, 24 h) required to attain the greatest possible biotransformation of isoflavone glucoside into aglycone form. Ideally, a fermentation period of 6 to 12 h would improve production efficiency and may be attainable using a different approach to the fermentation process. One method could be to ferment SPI/SG-soymilk with a combined culture of *Bifidobacterium animalis* Bb-12 and *Lactobacillus acidophilus* (AB culture) or *Lactobacillus delbrueckii* ssp. *bulgaricus*. A possible synergy between these two genera may enhance the enzymic transformation of isoflavone over a shorter incubation period. Then again, a combined culture may also increase organic acid production, causing the SPI/SG-soymilk to form into soy yoghurt. Alternatively, considering that *B. animalis* Bb-12 was able to reach a viable population of $>10^8$ viable cells per mL after 12 h (using a high inoculum level of ~7 $\log_{10}$ CFU per mL), but did not biotransform its maximum concentration isoflavone after this period, the SPI/SG-soymilk could possibly be treated with β-glucosidase during fermentation to assist in the hydrolysis of isoflavone glucosides. Additionally, an aspect which has not yet been investigated is the effect of fermentation by *B. animalis* Bb-12 on the oligosaccharide, aldehyde and phytate content of SPI/SG-soymilk. SG is a rich source of phytate and flatus-causing oligosaccharides. Phytate is most well known for its role in reducing the bioavailability of calcium. Hence, metabolising the phytate present in SPI/SG-soymilk may result in this product being a better source of calcium than conventional soymilk. Apart from the numerous health-related constituents that need further research, sensory studies on the degree of likeness and level of bitterness, beaniness, mouth-feel and acidity also need to be conducted on this SPI/SG-soymilk to assess its acceptability amongst consumers.

From clinical studies carried out in this project, there was a lack of strong evidence (supported by statistical significance) to indicate that the consumption of fermented SPI/SG-soymilk containing

viable bifidobacteria enhances the bioavailability of isoflavone in postmenopausal women (in terms of urinary percentage recovery), stimulates intestinal equol production, imparts positive effects on hormone profiles, and reduces the risk of cardiovascular disease. Even though the ingestion of fermented SPI/SG-soymilk appeared to reduce inter-individual variation in isoflavone absorption and urinary excretion, with a distinct linear dose-response amongst the group of women consuming fermented soymilk, this had no apparent positive influence on the physiological effects of fermented soymilk. There was a large inter-individual variability observed in the fermented soymilk group in terms of changes in LH, FSH, SHBG, lipid profiles, Lp(a) and bone resorption marker DPD, and it was equivalent to the variation shown amongst consumers of non-fermented soymilk and the placebo of casein-milk. Considering the importance of intestinal equol production suggested by earlier clinical studies, those women showing a positive physiological response to fermented and non-fermented soymilk supplementation may have consisted of the small proportion of women (typically ~30% of a study population) able to synthesise equol. Further analyses need to be conducted to determine whether there is a positive correlation between urinary equol excretion and maximal clinical response to fermented and non-fermented soymilk supplementation. On the other hand, the size of the study group and lack of statistical power may have been the reason for finding very little evidence of the positive health effects of fermented SPI/SG-soymilk. This study suggested that ingesting a fermented soymilk comprising ~50% of isoflavone in an aglycone configuration may be no different to consuming a non-fermented soymilk containing ~10% as aglycone. A fermented SPI/SG-soymilk comprising 100% of isoflavone in an aglycone form may be needed to enhance the effect of this product on markers of disease risk. Nevertheless, postmenopausal women consuming fermented soymilk containing ~50% isoflavone aglycone did show large reductions in the urinary excretion of bone resorption marker DPD, suggesting that such soymilk may be effective in preventing bone loss and osteoporosis. To support this potential bone-sparing effect on postmenopausal women, further analyses are required to determine whether the consumption of fermented SPI/SG-soymilk has a positive influence on markers of bone formation or a long-term study (1 to 2 years) to observe its effects on bone condition (bone mineral density and content).

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Appendix A
Method for Calculating the Concentration of β-Glucoside and Aglycone Isoflavone Isomers in Soymilk Analysed with HPLC

Four mixed isoflavone standards containing each β-glucoside and aglycone isomer at equal concentration (20, 50, 80 and 100 ng per 10 μL injection volume) and 600 ng of equilenin (internal standard) were analysed using HPLC at the beginning and end of each day of soymilk analyses. The chromatogram peak area responses for each of these isoflavone isomer standards were used to prepare a standard linear curve and equation to quantify the unknown concentration of isoflavone in each soymilk sample. A blank containing only 600 ng of equilenin was also analysed at the beginning and end of each day.

A.1 Preparation of a standard linear curve and equation

A.1.1 Calculated the Amount Ratio for each isoflavone standard at each level of calibration:

$$\text{Amount Ratio} = \frac{\text{Concentration of isoflavone in mixed standard (ng)}}{\text{Concentration of internal standard in mixed standard (ng)}}$$

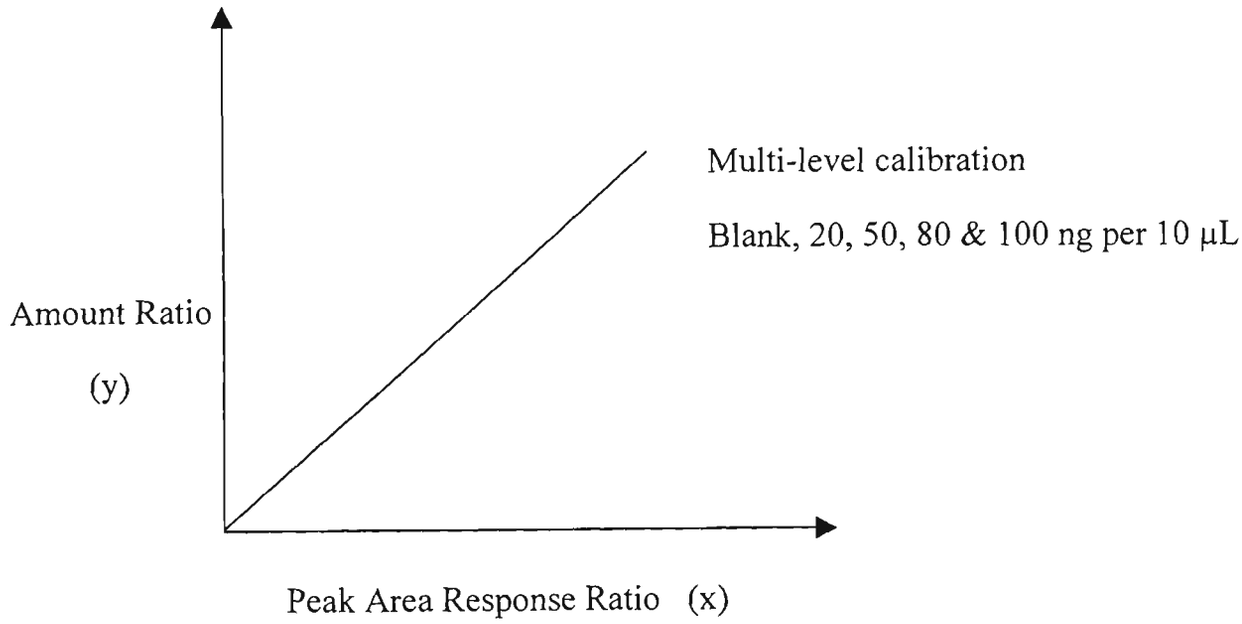
A.1.2 Calculated the Peak Area Response Ratio for each isoflavone standard at each level of calibration:

$$\text{Peak Area Response Ratio} = \frac{\text{Peak Area Response of isoflavone standard}}{\text{Peak Area Response of internal standard}}$$

A.1.3 Tabulated the Amount Ratio with its respective Peak Area Response Ratio for each isoflavone isomer at each level of calibration.

Amount Ratio (y-axis)	Peak Area Response Ratio (x-axis)

A.1.4 Plotted the Amount Ratio and Peak Area Response Ratio on an x, y-axis.



A.1.5 Determined the line of best fit and linear equation:

Amount Ratio = RFx (Peak Area Response Ratio) + c

Where:

RFx = gradient = Response Factor for values of x

c = y-intercept should be zero if the Blank injection showed no peak area responses at the specified isoflavone isomer retention times

The linear equation calculated above was used to determine the unknown concentrations of isoflavone in the soymilk samples.

A.2 Quantification of the unknown concentration of isoflavone in soymilk samples

A.2.1 Calculated the Peak Area Response Ratio for isoflavones detected in the soymilk sample chromatograms:

$$\text{Peak Area Response Ratio} = \frac{\text{Peak Area Response of detected isoflavone isomer}}{\text{Peak Area Response of internal standard}}$$

A.2.2 Calculated the Amount Ratio for isoflavones detected in the soymilk sample chromatograms using the equation in section A.1.5.

A.2.3 Calculated the amount of isoflavone in the 10 μL injection volume (ng) using the following equation:

Amount Ratio of detected isoflavone x Amount of internal standard (ng) = _____ ng per 10 μL

Where:

Amount of internal standard = 600 ng

A.2.4 Corrected the amount of isoflavone to represent that in the original freeze dried soymilk dissolved in 100 mL as follows:

_____ng per 10 μL x 100 =_____ng per 1 mL of resuspension =_____ng per 5 mL aliquot

_____ng per 5 mL aliquot x 20 =_____ ng per 1.00 g freeze dried soymilk

A.2.5 Calculated back to wet basis (ng per 100 mL soymilk) by using the volume of each soymilk sample freeze dried to 1.00 g (~25 mL) and multiplied by the appropriate freeze dry factor:

_____ng per 1.00 g freeze dried soymilk x (100 / volume of soymilk freeze dried to 1 g sample)

A.2.6 Expressed as mg isoflavone per 100 mL of soymilk by dividing by a factor of 10^6.

Appendix B
Method for Calculating the Concentration of Malonyl- and Acetyl-Glucoside Isoflavone Isomers in Soymilk Analysed with HPLC

B.1 Preparation of a standard linear curve and equation

Standard linear curves for each malonyl- and acetyl-glucoside isomer were devised using their respective isoflavone β-glucoside equivalent. For example, chromatogram peak area responses for standards of daidzin (20, 50, 80 and 100 ng per 10 μL injection volume) were used to construct a standard linear curve for malonyldaidzin and acetyldaidzin. Mixed standards of β-glucoside isoflavone isomers were analysed using HPLC at the beginning and end of each day of soymilk analyses, as described in Appendix A.

B.1.1 The peak area response (PAR) for each isoflavone β-glucoside standard was corrected according to the molecular weight (MW) of its respective malonyl- and acetyl-glucoside equivalent.

e.g.

PAR of daidzin x (MW of daidzin / MW of malonyldaidzin) = PAR of malonyldaidzin

B.1.2 Calculated the Amount Ratio for each isoflavone β-glucoside standard at each level of calibration.

$$\text{Amount Ratio} = \frac{\text{Concentration of isoflavone β-glucoside isomer (ng)}}{\text{Concentration of internal standard (ng)}}$$

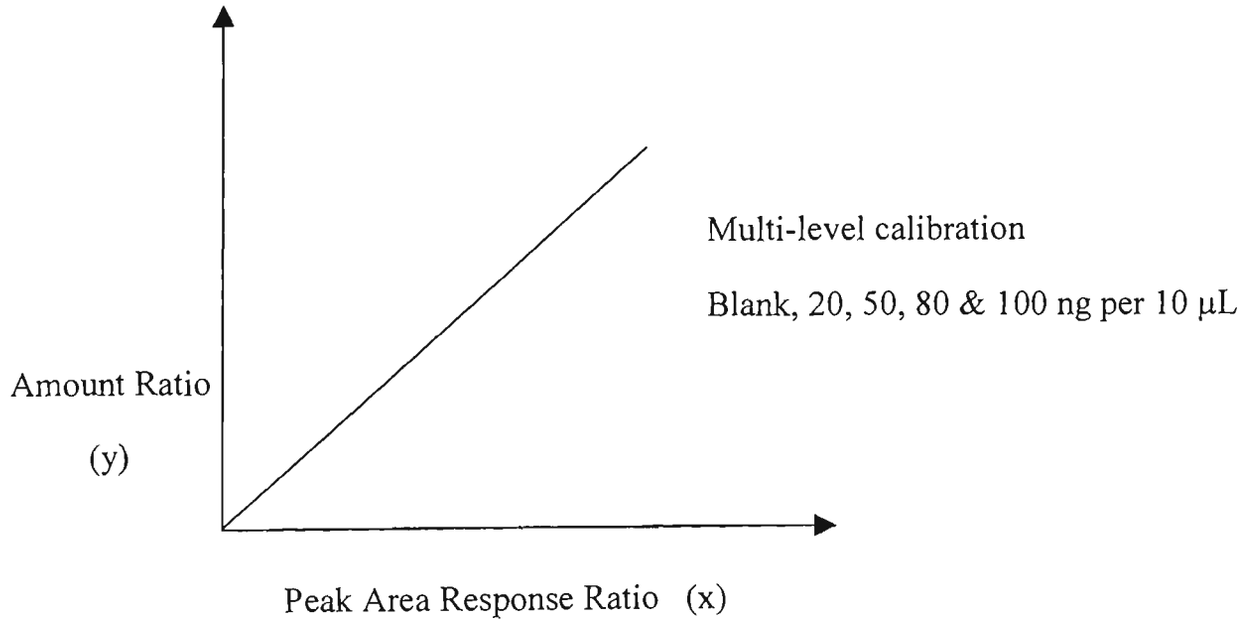
B.1.3 Calculated the Peak Area Response Ratio for each malonyl- and acetyl-glucoside isomer PAR calculated in section B.1.1.

$$\text{Peak Area Response Ratio} = \frac{\text{PAR of malonyl- or acetyl-glucoside isomer}}{\text{PAR of internal standard}}$$

B.1.4 Tabulated the Amount Ratio with its respective Peak Area Response Ratio for each malonyl- and acetyl-glucoside isomer at each level of calibration.

Amount Ratio (y-axis)	Peak Area Response Ratio (x-axis)

B.1.5 Plotted the Amount Ratio and Peak Area Response Ratio for each malonyl- and acetyl-glucoside isoflavone isomer on an x, y-axis.



B.1.6 Determined the line of best fit and linear equation:

Amount Ratio = RFx (Peak Area Response Ratio) + c

Where:

RFx = gradient = Response Factor for values of x

c = y-intercept should be zero if the Blank injection showed no peak area responses at the specified isoflavone isomer retention times

B.2 Quantification of the unknown concentration of isoflavone in soymilk samples

B.2.1 Calculated the Peak Area Response Ratio for each isoflavone malonyl- and acetyl-glucoside isomer detected in the soymilk sample chromatograms (retention times as per Setchell *et al.* 2001):

$$\text{Peak Area Response Ratio} = \frac{\text{PAR of detected malonyl- or acetyl-glucoside isomer}}{\text{PAR of internal standard}}$$

B.2.2 Calculated the Amount Ratio for each isoflavone malonyl- and acetyl-glucoside isomer detected in the soymilk sample chromatograms using the equation in section B.1.6.

B.2.3 Calculated the amount of isoflavone in the 10 μL injection volume (ng) using the following equation:

Amount Ratio of detected isoflavone x Amount of internal standard (ng) = _____ ng per 10 μL

Where:

Amount of internal standard = 600 ng

B.2.4 Corrected the amount of isoflavone to represent that in the original freeze dried soymilk dissolved in 100 mL as follows:

_____ng per 10 μL x 100 =_____ng per 1 mL of resuspension =_____ng per 5 mL aliquot

_____ng per 5 mL aliquot x 20 =_____ ng per 1.00 g freeze dried soymilk in 100 mL

B.2.5 Calculated back to wet basis (ng per 100 mL soymilk) by using the volume of each soymilk sample freeze dried to 1.00 g (~25 mL) and multiplied by the appropriate freeze dry factor:

_____ng per 1.00 g freeze dried soymilk x (100 / volume of soymilk freeze dried to 1 g sample)

B.2.6 Expressed as mg isoflavone per 100 mL of soymilk by dividing by a factor of 10^6.

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Health Information and Food Frequency Questionnaire

PLEASE USE BLOCK LETTERS AND A BLACK OR BLUE PEN

1. Personal Details

Name_____
Surname First Names

Address _____
Street Suburb

_____ **Phone no. (home)** _____
State Postcode **(mobile)**_____

Date of Birth _____/_____/_____ **Age** _____ **Ethnicity**_____
Day Month Year

Subject Code No. _____
(assigned by researcher)

2. Health Information

2.1. Are you post-menopausal (cessation of menstrual bleeding at least 1 year ago)?

Yes ☐ **No** ☐

2.2. How often are you involved in aerobic activity (vigorous sports, jogging, gym etc.) on a weekly basis? _____

2.2.1. Briefly mention some of the aerobic activities in which you participate?

_____

_____

2.3. Do you smoke? **Yes** ☐ **No** ☐

2.4. Have you taken any forms of hormone replacement therapy or antibiotics within the past 3 months? **Yes** ☐ **No** ☐
If the answer is yes, please provide details

_____

_____

_____

_____

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2.5. Do you regularly use prescription/non-prescription drugs (e.g. pain killers, allergy pills, sleeping pills)? **Yes** ☐ **No** ☐

If the answer is yes, please provide details

_____

_____

_____

_____

2.6. Do you suffer from any gastrointestinal disorders? **Yes** ☐ **No** ☐

If the answer is yes, please state the specific disorder/disease/ailment (optional)

_____

_____

_____

_____

2.7. Do you suffer from any food related allergies? **Yes** ☐ **No** ☐

If the answer is yes, please state which foods

_____

_____

2.8. Is your intake of alcoholic beverages in excess of 2 drinks per day (equivalent to 720 mL of beer per day, 240 mL of wine per day and/or 90 mL of hard liquor per day)?

Yes ☐ **No** ☐

3. Food Frequency and Dietary Information

3.1. How often do you consume soy-based foods? (please tick one of the categories below that best describes your intake)

Never	☐
Very rarely, 1 – 2 servings per 6 months	☐
Rarely, 1 – 2 servings per month	☐
On a weekly basis, 1 – 2 servings per week	☐
On a daily basis, 1 – 3 servings per day	☐

3.2. What are your reasons for consuming soy-based foods? (you may tick more than one box)

Low-fat protein alternative to meat derived protein	☐
To reduce my cholesterol intake	☐
Due to its potential disease preventative actions (e.g. breast cancer)	☐
Due to its potential roles as an estrogen replacement therapy	☐
Because it is healthy and I like the taste	☐
It is a traditional part of my diet	☐

Other reasons_____

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3.3. Which of the following soy-based food(s) do you incorporate as part of your diet?

Numerically rank each soy food according to the level of consumption, as follows:

Significant component of my soy intake	3
Moderate component of my soy intake	2
Rare component of my soy intake	1
I do not consume this soy food	0

Soy Foods	Rank
Soymilk made from whole soybeans	_____
Soymilk made from soy protein powders	_____
Tofu	_____
Miso	_____
Tempeh	_____
Soy-based cereals	_____
Soy yoghurts	_____
Soy burgers, sausages and other soy-based meat replacers	_____
Breads containing soy	_____
Other _____	_____
_____	_____

3.4. How often do you consume foods that contain live bacteria as a food ingredient? (please tick one of the categories below that best describes your intake)

Never	☐
Very rarely, 1 – 2 servings per 6 months	☐
Rarely, 1 – 2 servings per month	☐
On a weekly basis, 1 – 2 servings per week	☐
On a daily basis, 1 – 3 servings per day	☐

3.5. What are your reasons for consuming foods containing live bacteria as a food ingredient? (you may tick more than one box)

To soothe my gastrointestinal aches and pains	☐
To keep me regular (with respect to defecation)	☐
Due to its potential disease preventative actions (e.g. colon cancer)	☐
Low fat meal	☐
Because it is healthy and I like the taste	☐
It is a traditional part of my diet	☐

Other reasons_____

_____

_____

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3.6. Which of the following food(s) that contain live bacteria as a food ingredient do you incorporate as part of your diet?

Numerically rank each food according to the level of consumption, as follows:

Significant component of my intake of foods containing live bacteria	3
Moderate component of my intake of foods containing live bacteria	2
Rare component of my intake of foods containing live bacteria	1
I do not consume this food	0

Foods containing live bacterial culture	Rank
Dairy yoghurt	_____
Soy yoghurt	_____
Probiotic milks (e.g. Yakult)	_____
Other _____	_____
_____	_____

3.7. Which of the following best describes your diet? (please tick only one box)

Vegetarian or vegan (no foods derived from an animal)	☐
Ovo-Vegetarian (I include eggs as part of my diet)	☐
Lacto-Vegetarian (I include dairy products as part of my diet)	☐
Ovo-Lacto-Vegetarian (I consume both eggs and dairy products)	☐
Omnivorous Type 1 (balanced diet of all food groups)	☐
Omnivorous Type 2 (high intake of meat and dairy products)	☐
Omnivorous Type 3 (low intake of meat and dairy products)	☐

---

4. Signature

I give the researchers permission to use this information to assess my suitability for the study entitled **'Effects of Probiotic Bifidobacteria on the Metabolism of Soymilk Isoflavones in Post-Menopausal Women'**, and for analyses following this feeding study. I understand that the information given will be kept confidential and only available to each of the researchers mentioned on page 1 of the Plain Language Statement.

Signed_____ Date_____

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Research Project Involving Human Subjects

Consent Form

CERTIFICATION BY SUBJECT

I, ...

Of (address), ...

.....

have read and understand the Plain Language Statement.

I certify that I am voluntarily giving my consent to participate in the human dietary intervention study entitled:

according to the conditions described in the Plain Language Statement.

I am fully aware that the study is being conducted at Victoria University by:

Mr Dimitri Tsangalis (*BAppSc, PhD candidate*)
Professor Nagendra Shah (*BVSc, MSc, PhD*)
Dr Gisela Wilcox (*BMedSc, MBBS, FRACP, MAACB, FRCPA*)
Associate Professor Lily Stojanovska (*BSc, MSc, PhD*)

I certify that the objectives of the experiment, together with any risks to me associated with the procedures listed in the Plain Language Statement, have been fully explained by:

Mr Dimitri Tsangalis (*BAppSc, PhD candidate*)

I understand that this study will also involve the completion of a food record during each supplementation period.

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this study at any time and that this withdrawal will not jeopardise me in any way.

The researchers have also agreed not to reveal my identity and personal details if information about this project is published or presented in any public form.

Finally, I have been given a copy of the Plain Language Statement and signed consent form to keep for my own record.

Signed: ..... Date: .....

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Witness other than the researcher (print): .....

Signed: ..... Date: .....

Researcher's Name (print): .....

Signed: ..... Date: .....

Note: All parties signing the Consent Form must date their own signature.

Any queries about your participation in this project may be directed to the researchers (Dimitri Tsangalis ☎ 9216 8221; A/Professor Lily Stojanovska ☎ 9688 4233). If you have any queries or complaints about the way you have been treated, you may contact Ms. Elaine Cox, Victoria University Human Research Ethics Committee (☎ 9688 4705).

Subject Code: _____

Dietary Record Booklet

♦ Please exclude the following foods for the duration of the study:

1) Soy-based foods:
soy bread, soy breakfast cereals, soymilk, tofu, tempeh, miso, yuba, okara, soy cheese, green vegetable soybeans, natto, non-dairy soy frozen dessert and soy yoghurt.

2) Non-soy foods containing the following soy ingredients (usually found in pastries, brown and wholemeal bread, meat sausages):
soy flour, soy grits, soy protein concentrate, soy protein isolates, soy germ, whole soybeans and hydrolysed vegetable protein.

3) Foods containing the following:
chick peas, lentils, navy beans, alfalfa, mung bean and other bean sprouts

4) Probiotic milk products/supplements (eg. Yakult); and

5) Foods containing bacteria as a food ingredient (e.g. dairy yoghurts).

6) Alcoholic beverages

Soy sauce, soy butter, lecithin and soy oil consumption is permitted at any time during the study.

INSTRUCTIONS:

♦ Please use 1 blank dietary record page per day (front and back).
♦ Write in block letters and use a black or blue pen.
♦ Fill in the date in top right hand corner.
♦ Accurately describe the foods and/or beverages consumed.
♦ Note down the time of day you consumed the foods and/or beverages listed.
♦ Fill in the quantity of the food and/or beverage consumed during the meal. Please estimate if you are unaware of the accurate mass or volume of the food or beverage using common household measurements (for example: 1 cup; small, medium, large sized fruits and vegetables; number of teaspoons or table spoons)

DAY 1 **DATE:** _____

Time hr : min	**Food & Beverage Description, Serving Size & Number of Servings** (for uncommon foods also list their major ingredients)	**Quantity** (g or mL) **or common household measurement**

Appendix F
Method for Calculating the Concentration of Aglycone Isoflavone Isomers in Urine Analysed with HPLC

Six mixed isoflavone standards each containing daidzein, genistein and glycitein at equal concentration (50, 100, 150, 250, 350 and 450 ng per 50 μL injection volume) and 600 ng of benzophenone (internal standard) were analysed using HPLC at the beginning and end of each day of urine analyses. The chromatogram peak area responses for each of these isoflavone isomer standards were used to prepare a standard linear curve and equation to quantify the unknown concentration of isoflavone in each urine specimen. A blank containing only 600 ng of benzophenone was also analysed at the beginning and end of each day.

F.1 Preparation of a standard linear curve and equation

F.1.1 Calculated the Amount Ratio for each isoflavone standard at each level of calibration:

$$\text{Amount Ratio} = \frac{\text{Concentration of isoflavone in mixed standard (ng)}}{\text{Concentration of internal standard in mixed standard (ng)}}$$

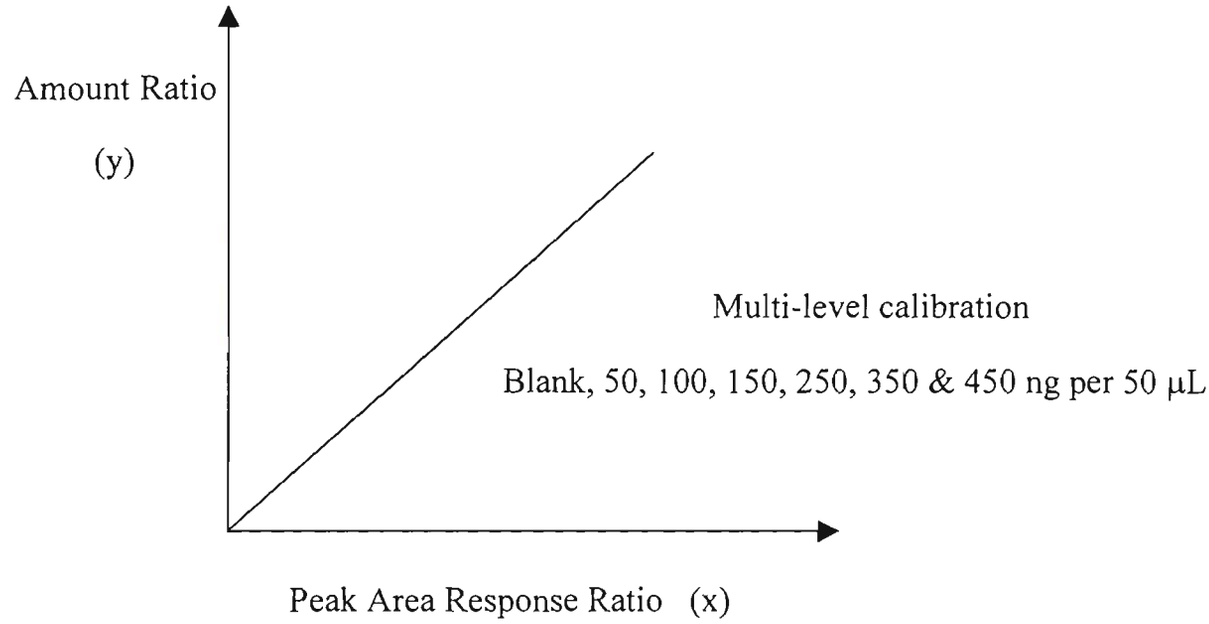
F.1.2 Calculated the Peak Area Response Ratio for each isoflavone standard at each level of calibration:

$$\text{Peak Area Response Ratio} = \frac{\text{Peak Area Response of isoflavone standard}}{\text{Peak Area Response of internal standard}}$$

F.1.3 Tabulated the Amount Ratio with its respective Peak Area Response Ratio for each isoflavone isomer at each level of calibration.

Amount Ratio (y-axis)	Peak Area Response Ratio (x-axis)

F.1.4 Plotted the Amount Ratio and Peak Area Response Ratio on an x, y-axis.



F.1.5 Determined the line of best fit and linear equation:

Amount Ratio = RFx (Peak Area Response Ratio) + c

Where:

RFx = gradient = Response Factor for values of x

c = y-intercept should be zero if the Blank injection showed no peak area responses at the specified isoflavone isomer retention times

The linear equation calculated above was used to determine the unknown concentrations of isoflavone in the urine specimens.

F.2 Quantification of the unknown concentration of isoflavone in urine specimen

F.2.1 Calculated the Peak Area Response Ratio for isoflavones detected in the urine specimen chromatograms:

$$\text{Peak Area Response Ratio} = \frac{\text{Peak Area Response of detected isoflavone isomer}}{\text{Peak Area Response of internal standard}}$$

F.2.2 Calculated the Amount Ratio for isoflavones detected in the urine specimen chromatograms using the equation in section F.1.5.

F.2.3 Calculated the amount of isoflavone in the 50 µL injection volume (ng) using the following equation:

Amount Ratio of detected isoflavone x Amount of internal standard (ng) = _____ ng per 50 µL

Where:

Amount of internal standard = 600 ng

F.2.4 Corrected the amount of isoflavone to represent that in the 20 mL urine specimen:

_____ng per 50 µL x 40 =_____ng per 2000 µL eluate =_____ng per 20 mL urine specimen

F.2.5 Calculated back to the total volume of urine excreted in 24 hours:

_____ng per 20 mL urine specimen x (total volume of urine excreted in 24 hours / 20) =

_____ng per 24-hour pooled urine specimen = _____ng of isoflavone excreted per day

F.2.6 Expressed as gram of isoflavone excreted per day by dividing by a factor of 10^9.

F.2.7 Expressed the amount of isoflavone excreted per day on a molar basis:

_____g of isoflavone / molecular weight of isoflavone isomer = _____mol per day

F.2.8 Expressed as µmol per day by multiplying by a factor of 10^6.