ASSESSING CALCIUM ABSORPTION FROM FORTIFIED SOYMILK AND FERMENTED FORTIFIED SOYMILK IN OSTEOPENIC POST MENOPAUSAL WOMEN

A thesis submitted for the degree of Doctor of Philosophy

By

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DEDICATED TO MY FAMILY

ABSTRACT

The overall objectives of this thesis were to assess the calcium absorption of calcium fortified soymilk (CFSM) compared to cows' milk in post menopausal women, and to investigate ways of optimising the calcium bioavailability from commercially available CFSM. The project consisted of two parts: *in vitro* studies and the *in vivo* clinical studies.

The objective of the *in vitro* studies was to enhance calcium bioavailability from CFSM by fermenting it with 6 strains of *Lactobacillus* spp. Calcium solubility, phytase activity, hydrolysis of phytic acid and isoflavone bioconversion were investigated. There was a significant increase ($P < 0.05$) in soluble calcium when pH decreased in the fermented CFSM. Most strains produced phytase during fermentation, with *L. acidophilus* ATCC4161 showing the highest activity. Phytic acid degradation was not observed. Fermentation of CFSM also increased ($P < 0.05$) the level of bioconversion of isoflavones into biologically active aglycones by ß-glucosidase activity. We also established that provided a heat treatment (90˚C for 30 min) was applied, labelling of fortificant after soymilk manufacture yields a similar tracer distribution pattern to when the fortificant is labelled prior to soymilk manufacture.

Our *in vivo* studies showed that fractional calcium absorption (α) from CFSM was comparable to cows' milk using a randomised single-blind acute crossover design study in 12 osteopenic post-menopausal women ($\alpha = 0.65 \pm 0.19$ and $\alpha = 0.66 \pm 0.22$ respectively, $P > 0.05$). When CFSM was fermented with *L. acidophilus ATCC*4962*,* the mean fractional calcium absorption from non fermented CFSM and fermented CFSM

was 0.64 (± 0.23) and 0.71 (± 0.29) respectively, a non-significant difference ($P = 0.122$).

Our results show that CFSM can be used as substitute to cows' milk as a calcium source. Fermentation did not enhance fractional calcium absorption; *in vitro* studies indicate its potential benefits towards calcium bioavailability and bone health.

CERTIFICATE

Professor Lily Stojanovska MSc, PhD

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Certificate

This is to certify that the thesis entitled "Assessing Calcium Absorption From Fortified Soymilk And Fermented Fortified Soymilk In Osteopenic Post Menopausal Women" submitted by Anne Lise Tang Fook Cheung in partial fulfilment of the requirements for the award of Doctor of Philosophy in Food Science and Nutrition at Victoria University is a record of bonafide research work carried out by her under my personal guidance and supervision, and the thesis has not previously formed the basis for the award of any degree, diploma or similar title.

Principal supervisor: _____ (Prof. Lily Stojanovska)

Date: _____

St Albans, Australia

DECLARATION

I, Anne Lise Tang Fook Cheung, declare that this thesis entitled "Assessing Calcium Absorption From Fortified Soymilk And Fermented Fortified Soymilk In Osteopenic Post Menopausal Women" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

_____ (Anne Lise Tang Fook Cheung)

Date: _____

St Albans, Australia

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Anne Lise Tang Fook Cheung

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PUBLICATIONS AND CONFERENCE PRESENTATIONS

Refereed research papers

(1) Tang, A. L., Shah, N. P., Wilcox, G., Walker, K. Z., & Stojanovska, L. (2007). Fermentation of calcium fortified soymilk with *Lactobacillus*: Effects on calcium solubility, isoflavone conversion, and production of organic acids. *Journal of Food Science*, 72(9), 431-436.

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Conference presentations

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(6) AL Tang Fook Cheung, G Wilcox, B Strauss, K Z Walker, J F Ashton, L Stojanovska. Comparing calcium absorption of fortified soymilk to cows' milk in osteopenic post menopausal women. 5th International Congress on Vegetarian Nutrition, Loma Linda University School of Public Health, California, USA, March 2008. (ORAL)

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BMC	Bone Mineral Composition
BMD	Bone Mineral Density
BMI	Body Mass Index (kg/m^2)
CEE	Conjugated Equine Oestrogen
CFSM	Calcium Fortified Soymilk
CFU	Colony forming units
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DXA	Dual-Energy X-ray Absorptiometry
ER	Oestrogen receptor
ERα	Oestrogen receptor α
ERβ	Oestrogen receptor β
FSM	Fermented soymilk
GS	Gas chromatography
HCL	Hydrochloric acid
HDL	High-density lipoprotein
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
IP6	Myo-inositol hexaphosphate
LAB	Lactic acid bacteria
LDL	Low-density lipoprotein
MRS	de Mann Rogosa Sharpe
NFSM	Non-fermented soymilk
RHHC	Rapid hydration hydrothermal cooling
RSM	Reconstituted skim milk
SERM	Selective oestrogen receptor modulator

SPI	Soy protein isolate
TCA	Trichloroacetic acid
UHT	Ultra high temperature
UV/VIS	Ultraviolet-visible

Chapter 1
Introduction to thesis

Recent studies have indicated that the soybean is more than a yielding source of high biological value protein (Zhou & Erdman, 2005). Soyfoods also contain relatively high amounts of phytochemicals such as genistein, daidzein and glycitein (Tsangalis, Ashton, Mcgill, & Shah, 2002). Epidemiological studies have suggested an association of soyfood consumption with lowered risks for prostate, breast and colon cancers; improved blood lipid profile; reduction of cholesterol; improved bone health; a delay in the onset of osteoporosis; reduced blood pressure; protection against heart disease; and an easing of menstrual and menopausal symptoms (Hoogenkamp, 2005; Tripathi & Misra, 2005). Studies done on soy protein and its isoflavones suggest that they reduce the risk of osteoporosis in peri and post menopausal women (Alekel, Germain, Peterson, Hanson, Stewart, & Toda, 2000; Chen, Ho, Lam, Ho, & Woo, 2003a; Potter, Baum, Teng, Stillman, & Erdman, 1998; Setchell & Lydeking-Olsen, 2003a).

Soy products are becoming an increasingly important food in the diet of many Australians, especially women. Women at the age of menopause need to consume foods rich in calcium to protect their bones against osteoporosis. Osteoporosis is a condition in which bones become fragile and brittle leading to a higher risk of fracture (breaks and cracks). It usually occurs when bone loses minerals such as calcium due to various reasons and the body cannot replace these minerals fast enough to keep bones healthy. The level of calcium in the body, however, depends not only on the amount of calcium in the food that is consumed but also on how well is the calcium absorbed into the body via the gut. One of the best sources of readily absorbed calcium is from cows' milk.

However, many women in Australia, particularly post menopausal women, drink soymilk in preference to cows' milk for various reasons.

Native soymilk contains significantly less calcium than cows' milk. Manufacturers have addressed this issue by fortifying the soymilk with extra calcium. But at present, it is not known how well this additional calcium is absorbed by the body. As post menopausal women need large amounts of calcium (1500 mg calcium per day) to prevent osteoporosis, it is important to determine whether they can obtain the calcium they need from fortified soymilk. Therefore, this research will address the question as to how much calcium from fortified soymilk is absorbed compared to cows' milk in post menopausal women.

So far only one study has compared the bioavailability of the calcium in fortified soymilk with that of calcium in cows' milk (Heaney, Dowell, Rafferty, & Bierman, 2000). However, the study was conducted on soymilk obtained in the American market, and not the Australian. The proposed study will assess the bioavailability of calcium from fortified soymilk in a product available on the Australian market.

Calcium absorption can be measured by various methods: balance method, concentration of calcium in blood serum, urine increment method where serum concentration of calcium rises and the calcium is transported into the urine, and another method is the tracer method. The tracer method is based on labelling a food source containing calcium with a tracer calcium isotope, usually radioactive or stable. The food source is then consumed and the level of tracer calcium is measured in the blood over a period of time to determine the rate of calcium absorption and the total amount of calcium absorbed (Nordin, 1997; Weaver & Heaney, 2006).

Stable isotopes and radioisotopes of calcium, referred to as tracers, and are used for this purpose. However, radioisotopes are cheaper compared to stable isotopes. The tracer methodologies, using either radioisotopes or stable isotopes, are very sensitive and precise because the natural background for the tracer (particularly if radioactive) is usually very low (Heaney, 2001). As stated above, this method requires the food source to be labelled with the selected tracers, namely: radioisotope (^{45}Ca, ^{47}Ca) or stable isotope (^{42}Ca, ^{44}Ca, ^{46}Ca).

There are two ways calcium food sources can be labelled; intrinsically, by which the isotope whether stable or radioactive is incorporated biosynthetically into the food, or extrinsically, where the tracer calcium is added directly to the food source (Fairweather - Tait, Fox, Harvey, Teucher, & Dainty, 2001) and the tracer calcium is allowed to mix and exchange uniformly. This study investigates an effective method of labelling of calcium fortified soymilk.

'Radioisotope' tracer method has been most widely used to assess the absorption of calcium by the body due to its established methodology and tends to be cheaper (Nordin, Morris, Horowitz, Coates, O'Loughlin, & Need, 2009; Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998). An alternative 'stable isotope' tracer method to assess calcium absorption is increasingly being practised in recent years (Bronner & Abrams, 1998; Moser-Veillon, Mangels, Vieira, Yergey, Patterson, Hill, & Veillon, 2001; Patterson & Veillon, 2001). However, the 'stable isotope' method tends to be very costly to perform, although it provides an accurate measure of calcium absorption and has no ethical constraints.

Calcium is one is the most indispensable minerals and it represents a large proportion of the elementary composition of the human body (Nordin, 1976, 1997). To enhance the bioavailability of calcium, not only should the amount of calcium ingested

be considered but also the other compounds it is being ingested with. Such components are the phytic acid, oxalic acids, phosphates, vitamin D, amount of cations and anions, and dietary components such as the amount of protein and fats (Nordin, 1976).

Calcium found in cows' milk is in the form of calcium phosphate, which binds with one of the milk protein, casein, to form a complex. Acidification of the complex causes the calcium phosphate to dissolve and the protein to precipitate (Damodaran & Paraf, 1997), thus allowing for optimum calcium bioavailability. There are no publications in the literature indicating to which protein the calcium in the soy binds or complexes with. Protein separation techniques such as electrophoresis will attempt to determine the calcium binding sites of the soy protein.

Other factors that may affect calcium absorption are: phytic acid, phosphorus, oxalic acid and dietary fibres. Phytic acid, also called myo-inositol hexaphosphate (IP6) or phytate, is known as the storage form of phosphorus in seeds, particularly abundant in cereal grains, oilseeds and legumes (soybeans) (Reale, Mannina, Tremonte, Sobolev, Succi, Sorrentino, & Coppola, 2004). Phytic acid is known to interfere with calcium bioavailability (Kumagai, Ishida, Koizumi, Skurai, & Kumangai, 2002) by chelating with several divalent mineral ions and making them unavailable for absorption (eg. Ca^{2+}) (Lonnerdal, Jayawickrama, & Lien, 1999), and it is known that the human gastrointestinal tract does not possess an endogenous enzyme, phytase, capable of hydrolysing phytic acid (Lonnerdal, Jayawickrama, & Lien, 1999).

Various studies have also shown that reducing the phytic acid content of soy can increase the absorption of minerals such as iron, zinc, copper, calcium and magnesium (Hurell, Reddy, Huillerat, & Cook, 2003; Kumagai, Ishida, Koizumi, Skurai, & Kumangai, 2002; Kumagai, Koizumi, Sato, Ishikawa, Suda, Sakurai, & Kumagai, 2004; Lonnerdal, Jayawickrama, & Lien, 1999). Many studies have contemplated to remove

phytic acid from soybean using acidic and alkaline reagents, cations, ultrafiltration and anion-exchange resins. The enzyme phytase from microbial origin, has been also used to enhance degradation of phytic acid to improve absorption of iron (Hurell, Reddy, Huillerat, & Cook, 2003).

Fermentation of food with probiotics has the potential benefit of increasing calcium absorption. Probiotic foods contain live microorganisms, such as lactic acid bacteria (LAB), which promote health by improving the balance of microflora in the intestines. Some of the commonly known probiotics belong to the *Lactobacilli* and *Bifidobacterium* genus. Strains of *Lactobacillus acidophilus, L. casei, L. plantarum, Bifidobacterium bifidum, B. longum* have been identified as having probiotic properties (Gibson, Rastall, & Fuller, 2003).

Lactic acid bacteria (LAB) are widely used in the production of fermented foods and beverage. Health promoting benefits of consumption of LAB have been known for several years. Some of the benefits include: improved tolerance to lactose; protection from gastroenteritis; prevention of coronary heart disease; colon cancer, irritable bowel syndrome; helps in improving digestion and gut function; and possible improvement in mineral bioavailability (Gibson, Rastall, & Fuller, 2003). However to ensure the health benefits, the fermented product has to contain viable amounts of probiotics ($> 10^6$ cfu/g) (Shah, 2000).

Studies have shown that during fermentation with certain strains of LAB, the enzyme phytase produced can help catalyse the stepwise hydrolysis of phytic acid, IP6, to myo-inositol via penta to monophosphates (Reale, Mannina, Tremonte, Sobolev, Succi, Sorrentino, & Coppola, 2004). Other research on cereal have shown that fermentation of dough using certain LAB can produce the enzyme phytase (Angelis,

Gallo, Corbo, McSweeney, Faccia, Giovine, & Gobbetti, 2003), therefore potentially can help to optimise calcium absorption.

Recent studies on calcium absorption and bone metabolism have shown that consumption of soy protein and isoflavones is associated with attenuation of bone loss, lowering of urinary calcium and increase of bone mineral density in post menopausal women (Atkinson, Compston, Day, Dowsett, & Bingham, 2004; Chen, Ho, Lam, Ho, & Woo, 2003b; Lydeking-Olsen, Beck-Jensen, Setchell, & Holm-Jensen, 2004; Spence, Lipscombe, Cadogan, Martin, Peacock, & Weaver, 2001). Soybeans and soy derived food contain phytoestrogens which are plant derived, phenolic compounds with a structural resemblance to human oestrogen, although they are not as biologically active. The biologically active, oestrogen-like isoflavone isomers are the aglycone configurations of genistein, daidzein, and glycitein (Setchell & Cassidy, 1999a). It has been shown that fermentation of soymilk with certain LAB can also increase the conversion of isoflavones into the biologically active aglycone form (Tsangalis, Ashton, Mcgill, & Shah, 2002; Tsangalis, Ashton, Stojanovska, Wilcox, & Shah, 2004).

The above indicates that fermentation of soymilk with LAB may have various potential benefits in enhancing the calcium bioavailability from fortified soymilk and provide a health promoting fermented fortified soy beverage that can be accessible to the general population with the aim of minimising osteoporosis.

The aims of the research were:

- To improve the calcium bioavailability of a commercially available fortified soymilk by fermentation using probiotics, such as LAB, by increasing the amount of soluble calcium, decreasing the phytic acid concentration and increasing the amount of aglycone form isoflavones.

- To compare the calcium exchangeability during intrinsic and extrinsic labelling of fortified soymilk, validating different methods of tracer labelling to assess calcium absorption and determine the calcium binding sites on the soy protein from the fortified soymilk using protein separation techniques such as electrophoresis.
- To assess the bioavailability of calcium from the commercially available fortified soymilk as compared to cows' milk in osteopenic post menopausal women using the radioisotope tracer method.
- To assess the effect of equol producing status on fractional calcium absorption.
- To assess the calcium bioavailability of the fermented fortified soymilk compared to the fortified soymilk and cows' milk in osteopenic post menopausal women.

Chapter 2 of this thesis deals with the literature review that highlights the functions of calcium, factors affecting calcium absorption, the consumption of soymilk and its potential benefits for post menopausal women, the use of probiotics for optimising calcium absorption from fortified soymilk and related *in vivo* studies. Chapter 3 reports on the fermentation of fortified soymilk with selected strains of LAB to increase calcium solubility. Chapter 4 focuses on the determination of the level of isoflavone conversion in fortified soymilk during probiotic fermentation to increase calcium absorption. Chapter 5 describes the phytase activity of the probiotic strains and phytic acid degradation. Chapter 6 compares the labelling methods of ^{45}Ca radioisotope in calcium fortified soymilk. Chapter 7 compares the fractional calcium absorption of calcium fortified soymilk to cows' milk in osteopenic post menopausal women. Chapter 8 determines the equol production status of the osteopenic post menopausal women.

Chapter 9 compares the fractional calcium absorption from fermented CFSM to non fermented CFSM. Chapter 10 focuses on reporting the overall conclusions and future directions of research.

Chapter 2
Literature review

2.1 Soybeans

Soybean has been cultivated as a food crop in China for five thousand years. For many thousands of years, cultivation remained restricted to Asia and soybeans only began to be grown in the West in considerable quantity from the beginning of the 20th century (Liu, 2004). The use of the soybean as food spread throughout the Asian continent during the early part of the last millennium, as people developed their own unique soyfoods based on tradition, climate, and local taste preferences. *Natto*, for example, is a product consisting of fermented soybeans, which was developed in Japan at least 3,000 years ago and continues to be popular in some regions today (Riaz, 2006). Soybean crops were first cultivated in small amounts in the United States in the late 18th century. Cultivation slowly expanded from the early 19th century as more Asians migrated to Europe and North America. During the 1920's, small companies with ties to the Seventh-Day Adventist Church began making tofu in the US and around the same time, soy flour started to gain popularity in both Europe and the United States as a low cost source of protein for the production of meat substitutes. During both World Wars, large amounts of soy flour were used to help offset meat shortages (Riaz, 2006).

A lot of progress has been made with respect to the cultivation, production, processing and end-use applications of soybeans. This recent revolution in soybean production and end-use processing has led to a rapid increase in soybean production on a global basis and to the development of various new uses of soybeans as food, feed and

industrial materials. Large scale development of the soybean crop and processing industry began in the US during the 1940's and 1950's, spurred on by a rapid increase in domestic and worldwide demand for both protein meal and vegetable oil. Brazil, Argentina and India have become major producers, as the world's demand for soy as food, vegetable oil and animal feed has continued to increase. Growth in China has been hindered by inefficiencies, with its production lagging behind most other major producers. Nevertheless, China is still the fourth largest soybean grower worldwide, and it was also the world's largest importer of soybeans in 2004 (Riaz, 2006).

Soybean (*Glycine max*) contains important macronutrients. Around 40% of the bean is relatively high biological value protein containing all essential amino acids. Around 20% of the bean is composed of lipids (about 15% saturated fat, 61% polyunsaturated fat and 24% monounsaturated fat) (Cederroth & Nef, 2009). Carbohydrates make up the remaining 30% of the bean, half comprising soluble carbohydrate (including sucrose, raffinose, and stachyose) and half being insoluble carbohydrate (dietary fibre).

The protein content of soybean can vary from 36% to 46% depending on the variety (Gracia, Torre, Marina, & Laborda, 1997). Storage proteins are predominant. These include the 7S globulin, β-conglycinin and the 11S globulin glycinin (which represents about 80% of the total protein content), as well as less abundant storage proteins such as the 2S, 9S and 15S globulins (Gracia, Torre, Marina, & Laborda, 1997). These proteins are largely responsible for the functional characteristics of soy protein ingredients. The glycinin and β-conglycinin content in soybeans differs among varieties depending on the growing environment and genetics (Rickert, Johnson, & Murphy, 2004). Glycinin is a hexamer (6 subunits), each of which contains an acidic and a basic polypeptide connected by a disulfide bond. At least five glycinin gene

products are known, providing multiple types of glycinin subunits and thus leading to great heterogeneity in mature glycinin macromolecules (Rickert, Johnson, & Murphy, 2004). β-conglycinin is a trimer, made up of three subunits α, α', and β which may exist in several combinations.

Soybean also contains many micronutrients and phytochemicals. These include isoflavones, phytate, soyaponins, phytosterol, vitamins and minerals (Cederroth & Nef, 2009). Saponins and phytosterols have been reported to have beneficial effects on blood cholesterol levels and on cholesterol absorption, while isoflavones may also have a beneficial role in lipid and glucose metabolism (Cederroth & Nef, 2009).

Almost all traditional soyfoods are made from whole soybeans. They can be classified into two categories: non-fermented and fermented. Non-fermented soyfoods include soymilk, tofu, soy sprouts, soymilk film (*yuba*), soy nuts, green vegetable soybeans and many others. Some fermented soyfoods include soy sauce, *miso*, *tempeh*, *natto* and others (Liu, 2004).

2.2 Soymilk

Soymilk consists of a water extract of soybeans, resembling dairy milk in both appearance and composition. Based on the method of preparation, soymilk is generally divided into traditional soymilk and modern soymilk (Liu, 2004). Traditional soymilk is made by a thousand-year-old method for small scale use. The process includes soaking and grinding the soybeans, filtering the milk from the solid products and heating. Soymilk made in this traditional way, usually has a limited shelf life, but also possesses a characteristic beany flavour and a bitter or astringent taste. All nutrients present come solely from the original soybeans (Liu, 2004).

Modern soymilk is produced by the use of modern technology and equipment to maximise taste, flavour, nutritional value and convenience. The techniques used may include decantation, formulation, fortification, homogenisation, ultra-high-temperature processing, aseptic packaging and automation (Liu, 2004). The resulting soymilk has a relatively bland taste with reduced beany flavour, and in most cases it is flavoured, sweetened, and/or fortified for better taste or nutrition, and packed for longer life. It should be noted that much of the soymilk available commercially is now prepared from soy protein isolate rather than from whole soybeans (Golbitz & Jordan, 2006). Manufacturers often find production from soybean isolate simpler and providing a more consistent product.

Soymilk usually contains 8-10% total solids depending on the water to bean ratio used in processing. Among the total solids found in soymilk, protein constitutes about 3.6-4.0%, fat 0-2%, and carbohydrates between 3-7%, (these values are taken across the range of commercially available soymilk). Thus, soymilk composition compares favourably with that of cows' milk. Additionally, soymilk is cholesterol free and lactose free (Liu, 2004). Genotypic changes in protein subunit composition however, can strongly affect the particle size distribution and the stability of soymilk. Heat treatment and homogenisation also have a significant effect on physio-chemical properties of soymilk and are used to improve particle size distribution (Nik, Tosh, Woodrow, Poysa, & Corredig, 2009). The active phytochemicals present in soybean are extracted during the manufacture of soymilk including isoflavones.

2.2.1 Fortification of soymilk

Cows' milk has such a high nutritional value that it is often described as 'a complete food'. Apart from high biological value protein, cows' milk is also rich in calcium, containing on average 120 mg/100mL. One potential negative effect resulting from a change from cows' milk to soymilk may be reduced calcium intake, since native soymilk is relatively poor in calcium (Heaney, Dowell, Rafferty, & Bierman, 2000). Manufacturers have addressed this issue by fortifying soymilks with calcium. Methods of fortification however may vary considerably between products (Heaney, Rafferty, & Bierman, 2005). Soymilk fortification appears to be an effective way to increase calcium intake and the total amount of absorbed calcium (Lopez - Huertas et al., 2006). This can have important health effects since calcium fortification of foods may aid in the prevention of osteoporosis (McCarron & Heaney, 2004). Adequate calcium intake has also been associated with reduced risk of hypertension, colon cancer, kidney stones and lead absorption (McCarron & Heaney, 2004).

Most of the calcium in milk is found as a colloidal caseinate-phosphate complex that is readily released during digestion, and hence has high bioavailability (Gueguen & Pointillart, 2000). Calcium bioavailability in soymilk, however, depends considerably on the choice of fortificant (Zhao, Martin, & Weaver, 2005). Several commercial calcium salts have been used for fortification of milk/soy beverages, including calcium carbonate, calcium chloride, calcium phosphate, tribasic calcium phosphate, calcium citrate malate, calcium lactate, calcium gluconate, calcium lactate gluconate and milk calcium (Singh, Arora, Sharma, Sindhu, Kansal, & Sangwan, 2007). The bioavailability of these salts may depend not just on their nature, but also on the nature of proteins present in soymilk, which can act as carriers during the absorption process. With some fortificants and some types of soymilk, the calcium may react strongly with soymilk

proteins, particularly after heat processing, leading to sedimentation and gelation. Stabilisers and emulsifiers have therefore been used to try to maintain calcium in suspension as well as to improve mouth-feel and appearance (Singh, Arora, Sharma, Sindhu, Kansal, & Sangwan, 2007).

Many foods apart from soymilk can be fortified with calcium, providing examples that may be relevant to soymilk manufacture. For example, calcium fortification of cows' milk followed by restoration of the initial pH, resulted in a calcium to phosphorus ratio that remained above one, ideal for calcium retention in the body (Singh, Arora, Sharma, Sindhu, Kansal, & Sangwan, 2007). Examination of two different types of fortification used to add calcium to orange juice however indicated that although each type of fortificant registered equivalent calcium content on the nutritional label, there was considerable difference in calcium bioavailability (Heaney, Rafferty, Dowell, & Bierman, 2005). Studies in the US suggest that the quality of calcium fortification in many beverages is uneven at best, so that consumers are likely to be misled with respect to the calcium benefit conferred by the beverage. For example, tracer equilibrium of calcium ranged from 25% to 79% in a range of soy and rice beverages tested (Heaney, Rafferty, & Bierman, 2005).

2.2.2 Soymilk consumption

There has been a significant growth in the market value of soymilk in the US, Canada and Australia between 2000 and 2008 (Table 2.1), reflecting consumers increasing interest in this food product. While market value of soymilk is higher in the US, Australia has experienced the greater overall growth. In 2008, the consumption of soymilk in Australia was around 3.4 L per capita compared with 1.1 L per capita in the

US and 1.3 L per capita in Canada. The soymilk market for retail grocery in Australia has been valued at $81.2M per year (Aztec data 2009, unpublished results)

Around 8% of Australians regularly purchase soymilk, while another 9% purchase soymilk infrequently. The majority of these consumers (76%) are women (Ashton, JF, 2009, unpublished results). The most popular Australian brand (consumed by 6.3% of households) is *So Good*, which is also the brand selected for use in our studies.

	Market value ($ million, US)				Market volume (million litres)			
Country	**2000**	**2008**	**Overall Growth (%)**	**CAGR† (2000 – 2008) (%)**	**2000**	**2008**	**Overall Growth (%)**	**CAGR† (2000 – 2008) (%)**
United States	257.7	677.7	163	13	146.5	336.8	130	11
Canada	28.7	101.4	253	17	15.3	43.9	187	14
Australia	27.4	126	360	21	17.6	74.3	322	20

†*CAGR, Compound annual growth rate*

Table 2.1 – Market value and market volume of the soymilk industry by country for 2000 and 2008

(Data extracted from Datamonitor Market Data Analytics, Datamonitor Asia-Pacific, Sydney, Australia)

2.2.3 Benefits of soy consumption

Soy supplements are becoming increasingly popular among women who are looking for health products to prevent bone loss and symptoms associated with menopause. Indeed many animal and human studies suggest that isoflavones such as those in soymilk have protective role (Levis, Strickman-Stein, Doerge, & Krischer). Results from one intervention study however, indicated that soy isoflavones had little favourable effect on body composition or physical performance in post menopausal women (Kok, Kreijkamp-Kaspers, Grobbee, Lampe, & van der Schouw, 2005).

2.2.4 Aldehydes in soy foods

The distinct odour associated with various soy foods is predominantly due to the presence of volatile carbonyl compounds, including aldehydes, ketones and alcohols (Liu, 1997). Among these volatile compounds, the aldehyde hexanal is primarily responsible for the 'beany' flavour of soy foods due to its extremely low flavour threshold (< 1 ppm). The formation of volatile compounds in soybeans results from chemical degradation of the lipid fraction catalysed by an enzyme naturally found in soybean cotyledons and known as lipoxygenase (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, in the presence of molecular oxygen, catalyse the hydroperoxidation of linoleic acid and other polyunsaturated lipids present in the bean that contain a *cis*,*cis*-1,4-pentadiene moiety. The primary products of this reaction are hydroperoxides (Liu, 1997).

2.3 Isoflavones

Isoflavones are plant compounds, belonging to a class known as phytoestrogens or plant estrogens. Soy and soy based foods are rich sources of isoflavones. Phytoestrogens also include compounds known as lignans, coumestans and fungal oestrogens. Plant oestrogens have a structural and a functional similarity to the human hormone oestrogen. They are present in a large number of plant foods and are often been consumed by humans and animals worldwide. Of all known plant oestrogens, the isoflavones have been studied most extensively. Soy isoflavones are the most important dietary source of phytoestrogens for humans, and the two major soy isoflavones are genistein and daidzein.

Genistein and daidzein are parent compounds which are metabolised from their parent plant precursors, biochanin A and formononetin. Isoflavones are inactive when present in the bound form as glycosides in plants; they then become active when the sugar residue is removed (Warren, Shortle, & Dominguez, 2002). Intestinal microflora induce fermentation of these plant compounds, and both metabolites and unfermented parent (aglycone) compounds are susceptible to absorption. Diadzein can be metabolised to equol or to O-demethylangolensin (O-Dma) while genistein may be metabolised to þ-ethyl phenol. Diadzein, genistein, equol and O-Dma are therefore the major phytoestrogens detected in the blood and urine of humans and animals (Tham, Gardner, & Haskell, 1998). Chemical structures of soy isoflavones daidzein, genistein and glycitein are shown in Figure 2.1.

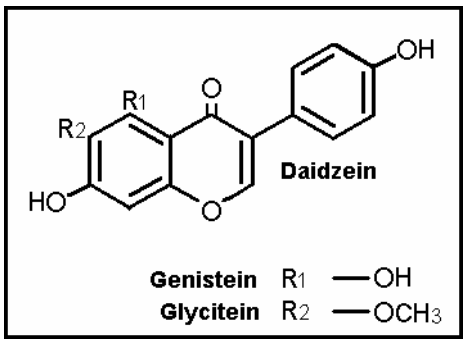


Figure 2.1 – Chemical structures of isoflavone aglycone isomers.

(Adapted from King & Bignell (2000))

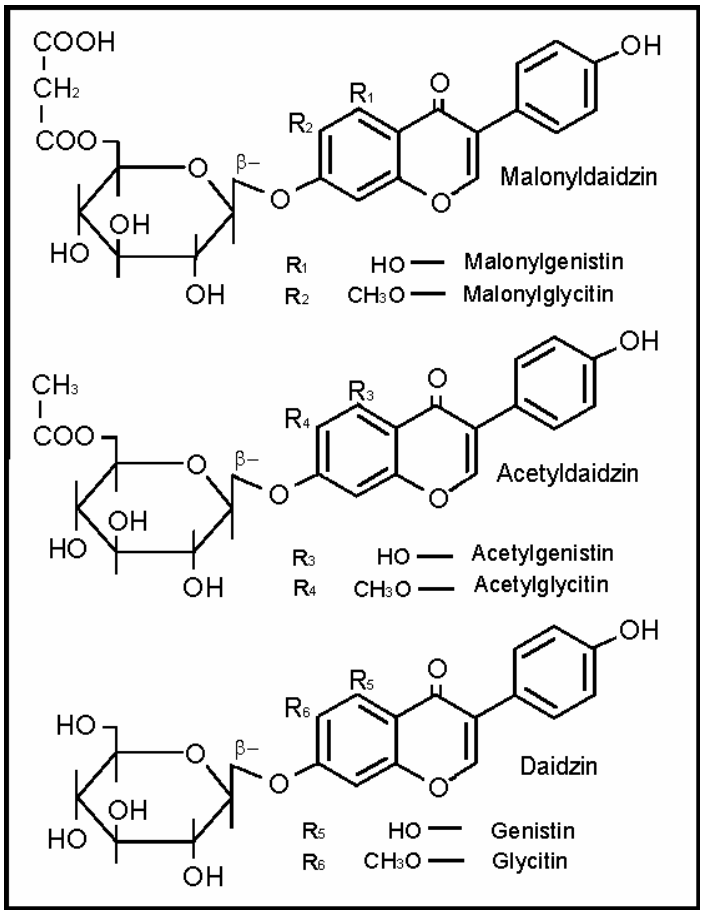


Figure 2.2 – Chemical structures of glucosidic isoflavone isomers.

(Adapted from (Ososki & Kennelly, 2003))

In the most recent study carried out on isoflavones in Australian commercial soymilks (40 varieties), Setchell & Cole (2003) found that the mean total isoflavone content of soymilks made from soy protein isolate (SPI), was 3.0 ± 0.6 mg per 100 mL. This 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. There was however, great variation in total isoflavone concentration amongst many whole bean soymilk brands, with values varying up to five-fold. With respect to shelf life, Setchell & Cole (2003) found no trend toward lower total isoflavone concentrations occurred with aging of ultra high temperature treated (UHT) whole bean and soy protein isolate (SPI)-based soymilks after 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)

The use of isoflavones from soy to modify the symptoms of oestrogen deficiency has been reviewed in many clinical trials. A significant inverse correlation has been reported between baseline hot flushes and the reduction in hot flushes achieved by isoflavone therapy, suggesting that isoflavone treatment may be effective only when the number of flushes experienced daily is relatively high (Messina & Hughes, 2003). This finding is also supported by a more recent study which found that the benefit of isoflavone supplementation was more apparent in women experiencing a high number of hot flushes per day (Howes, Howes, & Knight, 2006). A review of the literature suggests some evidence for the efficacy of soy preparations for menopausal symptoms including hot flushes and mood swings; although the heterogeneity and results, often

conflicting, owing to variation in soy preparations, dosage and duration of treatment precludes any definitive statement at present (Dog, 2005). Trial data however, indicates there are no serious safety concerns with the short term use of soy products, and that most evidence from clinical trials states that phytoestrogen from soy may alleviate menopausal symptoms (Huntley & Ernst, 2004).

One study in post menopausal women concluded that a soy-rich diet may be efficacious in increasing maturation of vaginal cells. Maturation indices may therefore be a useful marker for examining the efficacy of soy-based dietary interventions against menopausal effects and vaginal atrophy (Chiechi, Putignano, Guerra, Schiavelli, Cisternino, & Carriero, 2003). Soy germ isoflavone has also been found to exert favourable effects on vasomotor symptoms and on the lipid profile in post menopausal women, indicating that it may be useful as an alternative therapy to hormone replacement therapy (HRT) (Petri Nahas, Nahás Neto, De Luca, Traiman, Pontes, & Dalben, 2004). Isoflavones have been reported to increase nitric oxide breakdown and decrease endothelin-1 levels, thus improving vascular permeability in menopausal women (Cherma, Coomarasamt, & El-Toukhy, 2007). Soy isoflavones may aid in maintaining bone health after menopause. Soy isoflavone supplements were shown to moderately decrease levels of the bone resorption marker, urinary deoxypyridinoline (DPD), although they had no effect on bone formation markers, serum bone alkaline phosphatase (BAP) and serum osteocalcin (OC) in menopausal women (Taku, Melby, Kurzer, Mizuno, Watanabe, & Ishimi).

Soy isoflavones may also have a positive influence on cognitive function, which includes: reception (the acquisition, processing and classification of information); learning and memory, thinking and expression (as verbal or physical acts based upon thinking). As the learning and memory functions are pivotal, many studies on cognitive

function focus on these. Soy isoflavones appear to improve cognitive functions by mimicking the effects of oestrogen in the brain (Lee, Lee, & Sohn, 2005). Soy isoflavones, however, do not appear to be useful in the treatment of depression (de Sousa-Muñoz & Filizola, 2009).

Genistein specifically has been the phytoestrogen of greatest interest to researchers. Studies *in vitro*, indicate that genistein exerts both proliferative (oestrogenic) and antiproliferative (antioestrogenic) effects in humans (Murkies, Wilcox, & Davis, 1998; Sathyamoorthy, Wang, & Phang, 1994; Wang, Sathyamoorthy, & Phang, 1996). Genistein has been reported to be the most effective isoflavone for inhibiting cell growth of human prostate cancer cells (Chun, Kim, Lee, Cho, Kwon, Park, Jeong, Kim, & Kim, 2007). Studies on genistein, however, cannot be presumed to extend to daidzein as there appear to be important differences in the pharmacokinetics of the two isoflavones (Gardner, Chatterjee, & Franke, 2009).

A recent review (Wuttke, Jarry, & Seidlová-Wuttke, 2007) has questioned recommendations for soy isoflavone consumption since the review found the beneficial effects on climacteric complaints were very weak. In addition there were no clinical endpoint studies with the exclusive aim of investigating the effect of soy or soy isoflavone intake on incidence of mammary cancer and few examining their effects on cardiovascular events. Moreover, soy and soy isoflavones were considered likely to have only a mild bone protective effect.

There are numerous studies demonstrating the effects of isoflavones on bone health. The quantity and density of bone decrease with age after menopause. There is a negative relationship between age or time elapse since menopause and BMD at the spine or hip. This deterioration highlights the importance of early prevention of bone loss as soon as menopause is established. Animal studies indicate that a complementary

action of soy isoflavones and n-3 poly-unsaturated fatty acids (PUFAs) can contribute to the attenuation of bone mineral reduction in ovariectomised rats (Watkins, Reinwald, Li, & Seifert, 2005). In one study in women, an intake of 100 mg soy isoflavone per day was shown to stabilise bone loss in early menopause (Huang, Yang, Yang, Yang, Shieh, & Huang, 2006). In another study, consumption of isolated soy protein lowered loss of urinary calcium but was not associated with improved calcium retention, and soy isoflavones did not significantly affect calcium metabolism (Spence, Lipscomb, Cadogan, Martin, Wastney, Peacock, & Weaver, 2005).

2.3.1 Enzymic hydrolysis of isoflavones in soymilk

β-glucosidases hydrolyse isoflavone glucosides in soymilk, causing an increase in aglycone forms (Tsangalis, Ashton, Mcgill, & Shah, 2002). This is supported by the observation of maximum liberation of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside (a specific substrate for β-D-glucosidases) in soaking water at 50°C and pH 6.0 (Matsuura, Obata, & Fukushima, 1989). In a later study (Matsuura & Obata, 1993), it was found that incubating β-glucosidases (isolated from soybean cotyledon) in soymilk for 3 h at 45°C caused the hydrolysis of daidzin and genistin, increasing the concentration of daidzein and genistein. More recently, further studies used β-glucosidase to enhance the concentration of bioactive aglycones in soy ingredients (e.g. soy protein concentrate, soy meal and soy germ powder) via the hydrolysis of isoflavone glucosides (Otieno, Ashton, & Shah, 2006b; Tsangalis, Ashton, Stojanovska, Wilcox, & Shah, 2004). Figure 2.2 shows the chemical structures of glucosidic isoflavone isomers.

When *Lactobacillus delbrueckii* subspp. was incubated in MRS broth and soymilk, daidzin and genistin (each present at 50 μg per mL) were completely hydrolysed into their respective aglycones within 30 min due to the activity of β-glucosidase (Choi, Kim, & Rhee, 2002), an observation consistent with previous studies (Matsuura & Obata, 1993; Matsuura, Obata, & Fukushima, 1989).

A number of pharmacokinetic studies have investigated the bioavailability of isoflavones ingested in a bioactive aglycone-rich form compared to that of glucosidic isomers (Izumi, Piskula, Osawa, Obata, Tobe, Saito, Kataoka, Kubota, & Kikuchi, 2000; Setchell, Brown, Desai, Zimmer-Nechemias, Wolfe, Brashear, Kirschner, Cassidy, & Heubi, 2001b). Hutchins *et al*. (Hutchins, Slavin, & Lampe, 1995) fed men and women fermented soybean pieces (*tempeh*), rich in isoflavone aglycone, and found that urinary recovery of isoflavone was significantly greater than when the group consumed glucoside-rich isoflavones from 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 or 4 weeks of isoflavone aglycone and isoflavone glucoside intake. They found that plasma concentrations of daidzein and genistein were significantly greater in subjects consuming an aglycone-rich supplement ($P < 0.05$). In more recent studies, Setchell *et al*. (2001) reported that the bioavailability of daidzein and genistein was greater when they were ingested as β-glucosides rather than as aglycones based on the area under the curve (AUC) of the plasma appearance and disappearance concentrations. These acute studies differ from the longer studies of Hutchins *et al*. (1995) and Izumi *et al*. (2000), which found that the apparent bioavailability of isoflavone was greater when ingested as an aglycone. These researchers instead analysed urinary and plasma isoflavone profiles over a period of several days or weeks of isoflavone supplementation.

2.4 Phytic acid

Myo-inositol hexaphosphate (IP6) or phytic acid or phytate provides the major storage form of phosphorus in seeds and is particularly abundant in many cereals grains, oil seeds and legumes (Reale, Mannina, Tremonte, Sobolev, Succi, Sorrentino, & Coppola, 2004). Soybean contains 1% to 3% phytic acid (Isiguro, Ono, Nakasato, & Tsukamoto, 2005). Phytic acid is often reported as an anti-nutrient (Lopez, Leenhardt, Coudray, & Remesy, 2002) as it forms complexes with dietary minerals hindering their absorption and hence reducing their bioavailability (Lonnerdal, 2002; Messina, 1999). Many of the phytate-mineral complexes are insoluble and therefore, unavailable for absorption under normal physiological conditions. The chemical structure of phytic acid indicates a strong chelating potential. The effect on minerals is observed through the formation of both phytate-mineral and peptide-mineral-phytate complexes. Phytate forms a wide variety of insoluble salts with divalent and trivalent cations. The divalent cations (e.g., Zn^{2+}, Ca^{2+}, Mg^{2+}) form insoluble penta- and hexa-substitute salts (Weaver & Kannan, 2001). The insolubility of these complexes is regarded as the major reason for the reduced bioavailability of minerals seen in diets high in phytic acid since humans lack sufficient intestinal phytase to degrade these complexes (Weaver & Kannan, 2001). As much as 30%-97% of consumed phytic acid (0.3-3.7g/d) may remain undigested when it reaches the colon (Weaver & Kannan, 2001).

Several factors determine the effect of phytate on mineral availability: pH, size, valence of the mineral, phytate and mineral concentrations and ratios, and the food matrix. The latter includes the presence of enhancers and/or inhibitors and enzymes that may cause phytate hydrolysis. (Weaver & Kannan, 2001). As phytates are ionic in nature, they can react directly with charged groups of proteins containing a positively charged mineral ion such as calcium. The resultant phytate-protein and phytate-mineral-

protein complexes then decrease protein digestion and bioavailability (Reddy, 2001). Phytate can also bind with starch either directly by hydrogen bonding via a phosphate group or indirectly through proteins (Rickard & Thompson, 1997). Phytate-starch binding can then decrease starch solubility and digestibility. Phytates are heat stable so that they can withstand harsh field conditions, seed/grain transportation and long storage. Although food processing techniques such as soaking, cooking, milling, frying, roasting may decrease the phytate content, they do not completely eliminate phytate content (Reddy, 2001).

Phytate consumption can have beneficial effects on health. It has been reported that lower intake of inositol phosphate derivatives provide some protection against colon cancer (Shamsuddin, 1995, 2002; Steer & Gibson, 2002), arteriosclerosis, neural tissue and coronary heart disease (Fisher, Novak, & Agranoff, 2002; Jariwalla, Sabin, Lawson, & Herman, 1990). Phytates can also affect the quality of tofu (Isiguro, Ono, Nakasato, & Tsukamoto, 2005). It was reported that soymilk made from raw soymilk by heat had a phytate content of 0.252% ± 0.015% (Isiguro, Ono, Nakasato, & Tsukamoto, 2005).

2.4.1 Methods for analysis of phytate

The quantitative analysis of phytate (IP6) is based on precipitation with ferric chloride or purification using anion exchange chromatography. A disadvantage of these methods is the lack of specificity in distinguishing between IP6 and its degradation products (Skoglund & Sandberg, 2001). Inositol phosphates with three to five phosphate groups (IP3 – IP5) as well as IP6 are all nutritionally significant and it is therefore very important to have a reliable method for the determination of the individual inositol

phosphates. The development of ion-pair HPLC procedures and capillary electromigration methods, has been an important advance in making it possible to study IP6 and its hydrolysis products during processing and digestion (Skoglund & Sandberg, 2001).

The techniques for preparing samples used in most methods for determination of inositol phosphates include liquid and solid-phase extraction, centrifugation, freezing and thawing (to precipitate gelatinous agents and soluble proteins), and evaporation. For liquid extraction of inositol phosphates from foods or intestinal contents, an acid (hydrochloric acid HCl) can be used, while trichloroacetic acid (TCA) may be used in extraction of biological tissues as it removes both protein and lipids (Skoglund & Sandberg, 2001). The medium used in the ion exchange columns for solid-phase extraction can be either silica based anion exchange (SAX) or resin based anion exchange (AG 1-X8). The cleaning of ion exchangers is very useful for separating the ions from most impurities, removing inorganic phosphate and giving essential concentrations of inositol phosphates (Skoglund & Sandberg, 2001).

Precipitation methods are based on the principle that IP6 forms an insoluble stable complex with ferric ion in dilute acid. The phosphorous content in the precipitate can be determined after wet ashing or hydrolysis, giving a direct measure of the IP6 content (Skoglund & Sandberg, 2001).

Ion exchange methods are often used for processing a large number of samples. Although it is not rapid procedure, it is used for its simplicity and low cost. The technique is based on separating the inositol phosphates using a stepwise elution with HCl. The eluates are digested, inorganic phosphorus is measured and IP6 equivalent is calculated (Skoglund & Sandberg, 2001).

HPLC methods separate IP6 from inositol by using reversed phase octadecyl (C-18) stationary phases and aqueous potassium dihydrogen phosphate or sodium acetate mobile phases. One disadvantage of this method is elution of IP6 on the solvent front so that IP6 is only weakly retained on the column, resulting in extremely poor resolution (Skoglund & Sandberg, 2001).

^{31}P-Nuclear magnetic resonance (NMR) can be used to detect IP6 and its degradation products and also to determine the position of the phosphate groups (Skoglund & Sandberg, 2001).

2.4.2 Phytic acid hydrolysis

As phosphate groups are progressively removed from the inositol hexaphosphate (IP6), the mineral binding strength decreases and solubility increases (Jackman & Black, 1951). The degree of phosphorylation determines whether mineral absorption is enhanced (Weaver & Kannan, 2001). Hydrolysis may occur by both enzymic and non-enzymic methods. Phytate can also be hydrolysed by phytase which may occur endogenously in cereals and uncooked leafy vegetables (Weaver & Kannan, 2001).

Phytases are phosphatases that can utilise phytates as a substrate. Phytase enzymes are widely present in plants, microorganisms and animal cells. Industrially, plant and microbial phytases are utilised to degrade phytates in foods and feeds (Phillippy, 2001). Phytases will dephosphorylate IP6 to form free inorganic phosphate and inositol phosphate esters with a lower number of phosphate group. These are less able to influence mineral solubility and reduce intestinal uptake of micronutrients (Haraldsson, Veide, Andlid, Alminger, & Sandberg, 2005). Phytase activity was detected for the first time in *Bifidobacterium* spp. These bacteria were found to

dephosphorylate phytic acid to generate several myo-inositol phosphate intermediates (IP3-IP5) (Haros, Bielecka, & Sanz, 2005). Subsequently, several lactic acid bacteria (LAB) strains were found to be able to degrade phytic acid (Tamang, Tamang, Schillinger, Guigas, & Holzapfel, 2009). Moreover, *Bifidobacterium pseudocatenulatum* can produce phytases able to degrade phytate in synthetic medium and like probiotics, able to improve mineral absorption in the human intestine (Haros, Carlsson, Almgren, Larsson-Alminger, Sandberg, & Andlid, 2009).

2.5 Probiotics

The concept of a 'probiotic' was first introduced by Elie Metchnikoff, who observed that the consumption of fermented milk could reverse the putrefactive effects of the gut microflora (Metchnikoff, 1907). The term 'probiotic' was used to describe 'substances secreted by one microorganism which stimulated the growth of another' (Lilly & Stillwell, 1965). Parker defined 'probiotic' as 'organisms and substances which contribute to intestinal microbial balance'(Parker, 1974), while Fuller (Fuller, 1989) redefined the term to 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance'. Recently 'probiotics' were further defined as 'living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition' (Ross, Fitzgerald, Collins, & Stanton, 2002).

2.5.1 Characteristics of genus Lactobacillus

The genera *Lactobacillus* and *Bifidobacterium* contain a large number of bacterial species and strains that exhibit important properties in an applied context,

especially in the area of food and probiotics (Dellaglio & Giovanna, 2005). The genus *Lactobacillus* belongs to the LAB, a definition which groups gram-positive, catalase negative bacterial species able to produce lactic acid as the main end-product of the fermentation of carbohydrates (Dellaglio & Giovanna, 2005).

Lactobacilli are gram-positive, non-spore forming microorganisms occurring as rods or coccobacilli (Figure 2.3). They are strictly fermentative, microaerophilic and chemo-organotrophic, requiring rich media to grow. They are usually catalase negative, even if pseudocatalase activity can sometimes be present in some strains. They are also almost ubiquitous, being found in any environment where carbohydrates are available, including food (such as dairy products, fermented meat, sour doughs, vegetables, fruits and beverages); in the respiratory, gastrointestinal and genital tracts of humans and animals; and in sewage and plant material (Dellaglio & Giovanna, 2005).

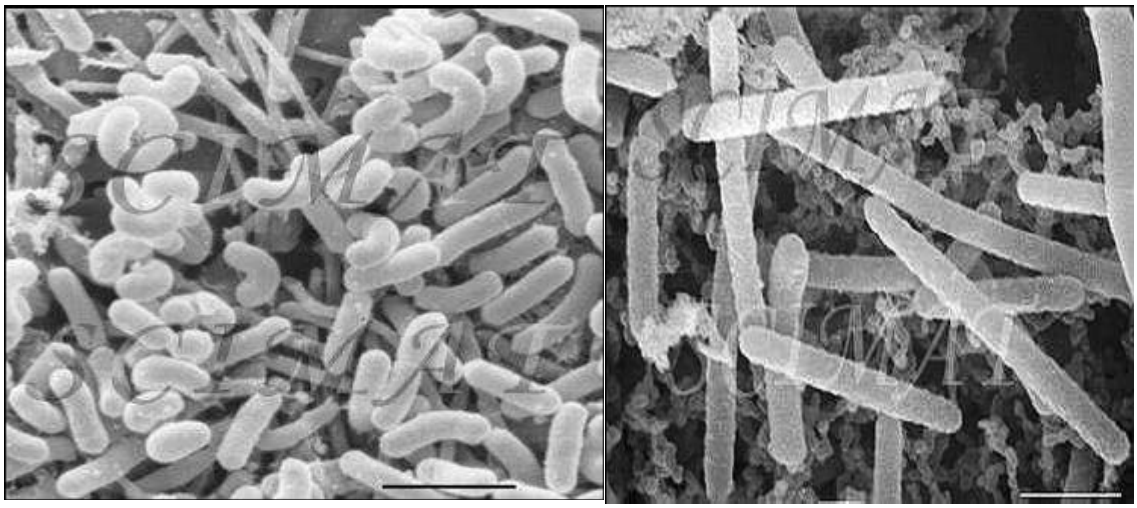


Figure 2.3 – Micrograph of *Lactobacillus casei* (bar 1 µm) and *Lactobacillus acidophilus* (bar 1 µm).

(Images are from SciMAT Photo Researchers, Inc.)

Some LAB species are aerotolerant, able to utilise oxygen through the enzyme flavoprotein oxidase, while others are strictly anaerobic. The growth of *Lactobacilli* is optimum at pH 5.5-5.8 and these organisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates (Axelsson, 2004). The genus is divided into three groups based on fermentation patterns namely: (1) homofermentative (producing > 85% lactic acid from glucose); (2) facultative heterofermentative (producing only 50% lactic acid from glucose plus considerable amounts of ethanol, acetic acid and carbon dioxide); and, (3) obligate heterofermentative species (producing DL-lactic acid, acetic acid and carbon dioxide). In early 2005, 97 species of the genus *Lactobacillus* had been recognised (Dellaglio & Giovanna, 2005).

Lactobacilli are widespread in nature and many species have found application in the food industry. The therapeutic roles of some *Lactobacillus* spp. have also been demonstrated. The ability of lactobacilli to convert lactose to lactic acid enables the successful treatment of lactose containing foods; therefore, cater for people who are lactose intolerant. *Lactobacillus* spp. also inhibits the growth of harmful putrefactive microorganisms by lowering the pH of the intestinal environment and through the ability of some strains to produce bacteriocins and other metabolic products such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2) and diacetyl (Ouwehand & Vesterlund, 2004). The bactericidal effect of H_2O_2 has been attributed to its strong oxidising effect on the bacterial cell. Some of the H_2O_2- producing reactions scavenge oxygen, thereby creating an anaerobic environment that is unfavourable for certain organisms. It has also been suggested that H_2O_2 production is particularly important for the colonisation of the urogenital tract by lactobacilli. Colonisation of lactobacilli has been found to decrease the acquisition of human immune deficiency virus (HIV)

infection, gonorrhoea and urinary tract infections (Fontaine, Claydon, & Tayler-Robinson, 1996). Formation of CO_2 creates an anaerobic environment and CO_2 itself has an antimicrobial activity (Ouwehand & Vesterlund, 2004). Diacetyl on the other hand, is thought to react with the arginine-binding protein of gram-negative bacteria thereby interfering with the utilization of this amino acid. Some strains of *Lactobacillus* such as *L. acidophilus*, *L. casei, L. helveticus, L. delbrueckii, L. lactis, L. plantarum, L. johnsonii, L. sake and L. curvatus* have been found to produce bacteriocins. These offer a more defined antimicrobial spectrum, ranging from related strains to a wide variety of gram positive and gram negative bacteria (Ouwehand & Vesterlund, 2004).

2.5.2 Application of probiotic organisms in food

Growing public awareness of diet related health issues has fuelled the demand for probiotic foods. A number of food products including yoghurt, frozen fermented dairy deserts, spray dried milk powder, cheeses, ice cream, coleslaw , freeze-dried yoghurt and fruit juices have been employed consistently to deliver probiotics to the consumer (Ong, 2007). One such food product considered in this thesis is fermented soymilk, also known as soymilk yoghurt, which is made in the same manner as cows' milk yoghurt. Thus pasteurised soymilk is inoculated with either *acidophilus, bifida*, or other suitable cultures and incubated until the culture has turned the soymilk into yoghurt. This product has a very similar taste to cows' milk yoghurt, and is also high in protein and a good source of isoflavones (Golbitz & Jordan, 2006).

2.5.3 Benefits of probiotics

A number of health benefits have been claimed for food products containing live probiotic bacteria. These benefits include alleviation of symptoms of lactose intolerance, treatment of diarrhoea, anticarcinogenic properties, reduction of blood cholesterol and constipation and stimulation of the immune system (Shah, 2000a; Shah & Wu, 1999). Emerging evidence also suggests that probiotic bacteria in the gut 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. Probiotics have also been applied successfully in the management of atopic eczema in infants (Schmid, Schlothauer, Friedrich, Staudt, Apajalahti, & Hansen, 2006). Furthermore, there is evidence from animal and human studies that suggests a moderate cholesterol-lowering effect associated with the consumption of fermented dairy products containing probiotic strains, possibly reducing the risk of cardiovascular diseases (Liong & Shah, 2005). Other claimed health benefits of probiotics are: positive effects on respiratory tract infections, on urogenital tract infections and inhibition of helicobacter pylori infection (Schmid, Schlothauer, Friedrich, Staudt, Apajalahti, & Hansen, 2006). Fermentation of cheese with probiotics may contribute to overall proteolysis during ripening and therefore result in the release of biologically active peptides such as angiotensin converting enzyme (ACE) – inhibitory peptides (Ong & Shah, 2008). Fermentation of probiotics in soymilk have shown to enhance the bioconversion of isoflavone to the biologically active aglycones (Otieno, Ashton, & Shah, 2006a; Tsangalis, Ashton, Mcgill, & Shah, 2002).

For the probiotic organisms to be of any value, they must be viable at the time of consumption. For this reason, changes in the numbers of viable bacteria during storage period should be known. It has been suggested that approximately 10^9 cfu per day of

probiotic microorganisms are necessary to elicit health effects (Goktepe, Juneja, & Ahmedna, 2006; Isolauri, Salminen, & Ouwehand, 2004; Tannock, 2005). Based on a daily consumption of 100 g or 100 mL of probiotic food, it has been suggested that a product should contain at least 10^7 cells per g or mL (Ross, Fitzgerald, Collins, & Stanton, 2002) in order to exhibit viable health effects, a level also recommended in Japan (Isolauri, Salminen, & Ouwehand, 2004). The selection criteria for probiotic organisms depend on a review of the literature and the scale of positive effects found for particular strains. Probiotic strains that have been shown to show promising aglycone converting properties from soymilk from a literature review would be preferably chosen and also taking in consideration of sensory acceptance of fermented soymilk.

2.6 Equol

Equol, [7-hydroxy-3-(4'-hydroxyphenyl)-chroman], is a non steroidal oestrogen that was first discovered in the early 1980s in the urine of adults who were consuming soy foods (Axelson, Kirk, Farrant, Cooley, Lawson, & Setchell, 1982). The molecule was then shown to be a key metabolite of daidzin, one of the main isoflavones present in most soy foods, and to be formed after intestinal hydrolysis of the soy isoflavone glycoside and subsequent colonic bacterial biotransformation through an intermediate dihydroxy equol (Figure 2.4) (Setchell et al., 2005).

Equol is not of plant origin but is exclusively a product of intestinal bacterial metabolism (Setchell, Brown, & Lydeking-Olsen, 2002). Isoflavone glycosides appear to be hydrolysed in the colon, as isoflavone cannot be absorbed by enterocytes. The gut flora is therefore critical in determining whether isoflavone aglycones are produced and resorbed to a sufficient degree or not. Diadzein must be reduced to the biologically

active equol. Genistein and daidzein play a role but the gut flora is crucial for the reduction of daidzein to equol (Wuttke, Jarry, & Seidlová-Wuttke, 2007).

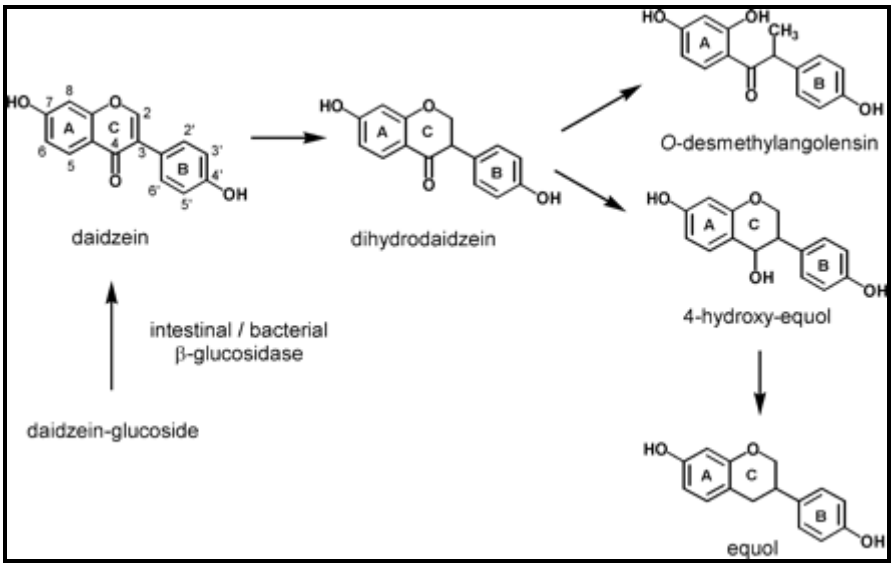


Figure 2.4 – Intestinal bacterial metabolism of the soy isoflavone daidzein to the isoflavone equol

(Source: (Rüfer, Glatt, & Kulling, 2006))

Although almost every animal species produces equol when fed a soy containing diet, only 20-35% of the Western adult population is capable of producing *S*-equol when fed soy foods or isoflavone supplements. A higher percentage (50-55%) of adults who live in Asian countries are equol producers and these populations regularly consume soy foods (Setchell & Cole, 2006). A few studies suggest that those who are equol producers experience greater health benefit from diets containing soy isoflavone suggesting that the metabolite *S*-equol has greater biological potency than either daidzein or genistein (Setchell, Brown, & Lydeking-Olsen, 2002). A study by Setchell & Cole (Setchell & Cole, 2006) suggests that an equol producer can be defined from urinary or serum equol concentrations and may require the consumption of soy foods or

isoflavone supplements containing daidzin or daidzein. Formation of S-equol may also occur relatively slowly and mainly in the distal intestine and colon. The response to a single serve of soy or isoflavone may not therefore be sufficient to define whether or not an individual is able to produce equol.

Equol is not normally present in the urine of most healthy adults in more than traces unless soy is regularly consumed. Its identification in human urine followed the fortuitous discovery that the rat, with its large caecum and abundance of microflora, was a large equol producer, in fact an 'equol-producing machine' (Setchell, Brown, & Lydeking-Olsen, 2002). The incubation of textured vegetable protein with cultured human faecal bacteria under anaerobic conditions confirmed the biotransformation of the soy isoflavone daidzein into equol (Setchell, Borriello, Hulme, Kirk, & Axelson, 1984). The main dietary origins of equol in humans are soy protein and soy foods (Setchell, Borriello, Hulme, Kirk, & Axelson, 1984) because these are the most abundant sources of the isoflavones daidzin and daidzein, the precursors of equol.

During the metabolism of equol, isoflavones in soy protein and most soy foods are conjugated to sugars. The β-glycosides are not absorbed and require hydrolysis for bioavailability and subsequent metabolism. Hydrolysis is extremely efficient and occurs along the entire length of the intestinal tract by the action of both the brush border membrane and the bacterial β-glucosidases (Setchell, Brown, & Lydeking-Olsen, 2002). Aglycones are then released and further metabolism takes place. Intestinal biotransformation includes dehydroxylation, reduction, C-ring cleavage and demethylation bacterial reactions that take place distally and presumably in the colon. Glycitin, the 6-methoxy analog of daidzin is found in high amounts in soy germ but is a minor component of soy foods. Glycitin is readily hydrolysed to release glycitein but the close proximity of the 6-methoxyl to the 7-hydroxyl stearically hinders its

demethylation. Therefore, glycitein is not converted to any appreciable extent to daidzein and is therefore not a precursor of equol (Setchell, Brown, Desai, Zimmer-Nechemias, Wolfe, Brashear, Kirschner, Cassidy, & Heubi, 2001b; Zhang, Wang, Song, Murphy, & Hendrich, 1999). The formation of equol from daidzein occurs via a pathway that involves the formation of the intermediate dihydrodaidzein. Studies have shown that equol is formed from daidzein and not genistein (Setchell et al., 2003).

2.6.1 Properties of equol

Unlike the isoflavones from soy, daidzein and genistein, or those in clover, formonetin and biochanin A, equol is unique in having a chiral centre due to the lack of a double bond in the hetrocylic ring, resulting in two distinct optically active isomers occur. These *R*- and *S*-isomers differ conformationally which undoubtedly influences ligand binding in the cavity of the dimerised estrogen receptor (ER) complex (Setchell, Brown, & Lydeking-Olsen, 2002). Equol is a weak estrogen, whereas its precursors daidzein and formonetin are inactive (Shutt & Braden, 1968). Metabolism has to be considered in assessing estrogenic potency because formonetin and daidzein act as proestogens, they are activated to greater potency by the action of bacterial flora in converting these to equol (Setchell, Brown, & Lydeking-Olsen, 2002). Uterine weights of immature rats killed after subcutaneous injection of (±) equol show that equol is more than twice as estrogenic as genistein in this model when allowing for the fact that half of the injected dose is an inactive enantiomer (Setchell, Brown, & Lydeking-Olsen, 2002).

Genistein has a high binding affinity for estrogen receptor β (ERβ), whereas daidzein and formonetin bind poorly (Kuiper, Lemmen, Carlsson, Corton, Safe, Van Der Saag, Van der Burg, & Gustafsson, 1998). The binding affinity of equol for human

ERα and ERβ is similar to that of genistein, although equol induced transcription more strongly than any other isoflavone, especially for estrogen receptor α (ERα) (Morito et al., 2001). Diadzein in contrast, showed poor affinity and transcriptional activity in these *in vitro* systems suggesting it could be advantageous to convert daidzein to equol to enhance its estrogenic potency *in vivo*. Equol can bind to sex hormone binding globulin (SHBG) and competitively inhibits estradiol and testosterone binding in a dose dependent manner (Martin, Haourigui, Pelissero, Benassayag, & Nunez, 1996). Equol also possesses other properties of relevance to cellular function. As a polyphenol, it shares with flavonoids the ability to be a hydrogen/electron donor and therefore can scavenge free radicals. Equol also has the greatest antioxidant activity of all the isoflavones tested when measured *in vitro* in the ferric reducing ability of plasma (Setchell, Brown, & Lydeking-Olsen, 2002).

2.6.2 Clinical effects of equol

It is important to define individuals by their 'equol-producing' status prior to an enrolment into a related dietary intervention study. This status can be determined via 'bacterio-typing' individuals on the basis of whether they possess the bacterial flora necessary to produce equol. It is easily achieved by mass spectrometry of plasma or urinary analysis (Setchell, Brown, & Lydeking-Olsen, 2002). The importance of determining equol producing status is illustrated by one study which showed that women who were 'equol producers' (45% of total participants) showed significant mean increases of 2.4% for BMD and 2.8% for BMC in the lumbar spine, compared with increases of only 0.6% and 0.3% respectively observed in women who did not produce equol (Lydeking-Olsen, Jensen, Setchell, Damhus, & Jensen, 2002). In another study,

35% of participants were ‘equol producers’ and after consuming 5 serves of soy per day for 5 weeks or a similar amount of dairy food, soy consumption significantly lowered plasma total cholesterol by 8.5%, LDL cholesterol by 10%, LDL/HDL cholesterol ratio by 13.5%, triglycerides by 21% and lipoprotein(a) by 11%, but only in the ‘equol producers’ group (Setchell, Brown, & Lydeking-Olsen, 2002).

2.7 Menopause

Menopause is the time of life when menstrual cycles cease, and is caused by reduced secretion of the ovarian hormones oestrogen and progesterone (Nelson, 2008). Natural menopause is recognised after 12 consecutive months without menstrual periods that are not associated with other causes (Castelo-Branco, Palacios, Calaf, Vázquez, & Lanchares, 2005). Menopause is a natural event for women and the menopausal transition (the time of an increase in follicle-stimulating hormone and increased variability in cycle length) usually begins when women are in their mid to late 40s. Individual experiences vary and women usually seek medical advice for the management of the associated symptoms. As oestrogen has a regulatory role for many organs, the age related deficit of this hormone triggers many physiopathological reactions. These include symptoms of degenerative processes, such as hot flushes, mood swings, acceleration of arteriosclerosis, accelerated bone loss and skin aging, which may impair the health and quality of life for many menopausal and post menopausal women (Miquel, Ramírez-Boscá, Ramírez-Bosca, & Alperi, 2006). Menopause is followed by an immediate fall in bone mass and density which has been detected within one year of cessation of menstruation in both cross sectional and longitudinal studies and at both peripheral and central sites (Nordin, Need, & Morris, 1993). It is most rapid

in the first few years after the menopause, when it is mainly trabecular, and then slows down, but the process continues to the end of life (Nordin, Need, & Morris, 1993).

Hormone replacement therapy (HRT) is the most effective treatment in reducing hot flushes for menopausal women (Utian, Archer, & Bachmann, 2008). However, not all women can, or prefer to, take HRT. Despite the potential health benefits of oestrogens commonly conjugated equine oestrogen (CEE) in the United States, and 17β-oestradiol in Europe, oestrogens have the disadvantage of being tissue agonists for breasts and endometrial tissue. Adding progestin to oestrogen (combined HRT) may increase the incidence of breast and ovarian cancers (Million Women Study Collaborators, 2003, 2007) but also poses unwanted side-effects, e.g.. vaginal bleeding, and bloating and depression. Side-effects such as cholelethiasis, breast tenderness and mood changes may result from conventional HRT (Warren, Shortle, & Dominguez, 2002). When the Women's Health Initiative (WHI) study was discontinued owing to unanticipated increases in risk for breast cancer, stroke, heart attack and blood clots among women taking oestrogen plus progestin (Rossouw, Anderson, & Prentice, 2002), the search for alternative treatments that were perceived to offer beneficial effects with less risks became predominant. Thus, alternative therapies, which include natural products such as phytoestrogen (including soy isoflavones) and herbs (black cohosh, red clover, dong quai), may become attractive options for some women. These alternative therapies may provide relief of vasomotor menopausal symptoms, primarily hot flushes and possibly mood disorders (Geller & Studee, 2005; Wong, Lim, Luo, & Wong, 2009), thus improving quality of life at mid-life and beyond.

Phytoestrogens are weak oestrogens found concentrated in soybeans and they are consumed in traditional soy food, soymilk, isolated soy protein added to food during its processing as a beverage. Extracted phytoestrogens are also used as dietary supplements

by peri and post menopausal women as an alternative to HRT (Miquel, Ramírez-Boscá, Ramírez-Bosca, & Alperi, 2006).

2.7.1 Peri menopause and post menopause

The peri menopause is defined as the period immediately prior to menopause and the first year after menopause. Thus, peri menopause includes the menopausal transition and overlaps the first 12 months of the post menopausal period. Post menopause is not recognised until after 12 months of amenorrhoea (Castelo-Branco, Palacios, Calaf, Vázquez, & Lanchares, 2005). Post menopause is known to be an important phase in the development of osteoporosis, associated fractures and further complications (Huang, Yang, Yang, Yang, Shieh, & Huang, 2006).

2.8 Calcium

Calcium is a mineral that accounts for 1% to 2% of the adult human body weight and plays a vital role in the development and maintenance of a healthy skeleton (Gueldner, Grabo, Newman, & Cooper, 2008). The great majority (99%) of body calcium is found in bones and teeth providing mechanical rigidity to the body. The divalent cation, or ionised, calcium – (Ca^{2+}) is also critical in many aspects of normal human health, playing vital roles in fertilisation, metabolism, blood clotting, nerve impulse conduction, muscle contraction, structure of the bony skeleton, and cellular communication (Awumey & Bukoski, 2006).

The primitive function of the skeleton is to serve as a source and as a sink for calcium and phosphorus, i.e., as a reserve to offset shortages and as a place for safely storing dietary surpluses, at least after periods of depletion (Heaney, 2005). This feature can be observed when animals placed on low calcium intakes, show reduced bone mass

as calcium is needed to maintain near constancy of calcium levels in the extracellular fluids. This activity is mediated by parathyroid hormone (PTH) and involves actual bone destruction, not leaching of calcium from bone (Heaney, 2005).

2.8.1 Calcium requirement

Since 99% of the total body calcium is located in the skeleton, the biology of this element must be central to the understanding and management of osteoporosis (Nordin, Need, & Morris, 1993). Bone balance and calcium balance are synonymous; if the bone balance is negative, then external calcium balance must also be negative and vice versa (Nordin, Need, & Morris, 1993). The calcium requirement of an adult is the calcium intake at which external balance is zero, a value governed by the relationship between calcium intake, absorption and excretion (Nordin, Need, & Morris, 1993). Previous reports have shown the calcium requirement was calculated from calcium balances in normal subjects on a range of calcium intakes. In 212 such balances on 85 subjects published in the literature, the mean value was found to be 550 mg/day (Nordin, Need, & Morris, 1993). This value was based largely on American balance studies conducted on young adults and may not apply to populations where protein and sodium intakes are lower or higher.

The net absorbed dietary calcium rises steeply as a result of active transport, but then approaches linearity with a gradient of about 6% representing absorption by diffusion. The slope of urinary calcium from the diet is also about 5%-6% and clearly reflects the diffusion component of the absorption slope. At intakes below 550 mg, urine calcium exceeds absorbed calcium. At higher intakes, absorbed calcium exceeds urinary calcium by an amount which approximates to dermal losses. At zero calcium

intake, there is a negative calcium balance of about 200 mg daily due to the loss of around 100 mg in the faeces and 100 mg in the urine. As calcium intake is increased, net absorbed calcium increases but the faecal and urinary calcium also rise until absorbed calcium matches urinary calcium when they both reach about 140 mg/day. At this point, the calcium intake is about 550 mg/day (Nordin, Need, & Morris, 1993). Owing to individual variation, the allowance needed to meet the requirements of 90% of the population is about 800 mg, which forms the basis of the recommended dietary allowance (RDA) of calcium in Australia (National Health & Medical Research Council, 1991).

2.8.2 Factors affecting calcium absorption

Several nutritional factors influence calcium requirements. A few nutrients create interactions, tending to have negative impacts on the calcium economy. The principal interacting nutrients are sodium, protein, caffeine and fibre (Heaney, 2005). Fibre and caffeine influence calcium absorption and typically exert relatively minor effects, whereas sodium and protein influence urinary excretion of calcium and can be of much greater significance for the calcium economy when calcium intakes are low (Heaney, 2005). Only 11% of the variance in calcium balance is explained by differences in actual intakes, absorption efficiency explains about 15% and urinary loss accounts for more than 50% (Heaney, 2005).

2.8.3 Influences on intestinal absorption of calcium

2.8.3.1 Fibre

The effect of fibre is variable and generally small. Many kinds of fibre have no influence at all on absorption, such as soluble fibre in green, leafy vegetables. In contrast, the insoluble fibre in wheat bran reduces absorption of co-ingested calcium (Heaney, 2005). Phytate and oxalate can also reduce the availability of any calcium contained in the same food; unlike bran, phytate and oxalate generally do not affect co-ingested calcium from other foods.

2.8.3.2 Caffeine

Although caffeine is often considered to have deleterious effect on the calcium economy, it has the smallest effect of the known interacting nutrients. A single cup of brewed coffee causes deterioration in calcium balance of approximately 3 mg, mainly by reducing calcium absorption of calcium and this loss is more than adequately offset by 1-2 tablespoons of milk (Heaney, 2005).

2.8.4 Influences on renal conservation of calcium

2.8.4.1 Protein and sodium

Protein and sodium can increase urinary calcium loss across the full range of their intakes, from very low to very high; thus the concern is not solely on the harmful effects of an excess of these nutrients. Sodium and calcium share the same transport system in the proximal tubule, and every 2,300 mg of sodium excreted by the kidney pulls 20 to 60 mg of calcium out with it.

Every gram of protein metabolised in adults additionally causes an increment in urine calcium loss of about 1 mg (Heaney, 2005). One study showed that urine calcium remained high even in adolescent girls on calcium intakes too low to permit bone gain (Matkovic, Fontana, Tominac, Goel, & Chestnut, 1990). The main determinant of urinary calcium in young women is sodium intake, not calcium intake (Matkovic, Fontana, Tominac, Goel, & Chestnut, 1990). Differences in protein and sodium intake among national groups are perhaps part of the reason that studies in different countries have shown strikingly different calcium requirements (Heaney, 2005).

2.8.4.2 Phosphorus

Phosphorus is believed to reduce calcium absorption but there is no clear evidence on this matter. An analysis of 567 metabolic balances performed in healthy middle-aged women studied on their usual diets, indicated that variation in phosphorus intake over a nearly six-fold range had no detectable effect on calcium absorption efficiency (Heaney, 2000). In adults, variation in the calcium to phosphorus ratio (Ca:P) from 0.2 to more than 2.0 are without effect on calcium balance, at least so long as adjustments are made for calcium intake (Heaney, 2000). Phosphorus does not depress urinary calcium loss and elevates digestive juice secretion of calcium by approximately equal amounts, with little to no net effect on balance (Heaney & Recker, 1994).

2.8.5 Calcium sources

Foods are the calcium sources for humans. Food items that provide more than 100 mg of calcium per serving are limited to dairy products, greens of the mustard family, calcium-set tofu, sardines, and some nuts (especially hazelnut and almonds).

Smaller amounts of calcium are ubiquitous in many leafy vegetables; while with the exception of shellfish, calcium levels are low in most meats, poultry and fish (Heaney, 2005). The calcium present in beans is only about half as that available as the calcium of milk and the calcium of high oxalate vegetables(such as spinach and rhubarb) is almost completely unavailable (Heaney, 2005).

In Western countries, diets that are low in dairy products are generally also low in calcium (~300-400 mg). Consequently, there has been an increasing trend of fortification of food with low levels of calcium. In the US, fortified foods range from juice to bread to potato chips to rice. Satisfactory bioavailability still needs to be determined for those fortified foods; a soy beverage fortified to the calcium load of cows' milk was shown to deliver only 75% of the calcium delivered by cows' milk (Heaney, Dowell, Rafferty, & Bierman, 2000). Recent entries into the soy and rice beverage markets as well as several of the calcium fortified orange juices exhibited even poorer physical characteristics (Heaney, Rafferty, & Bierman, 2005).

The main calcium supplement available in the US market is calcium carbonate formulated so that it disintegrates in the gastric juice (Heaney, 2005). Calcium carbonate is well absorbed and generally well tolerated. Calcium citrate and calcium malate are also good sources but can be more costly (Heaney, 2005).

2.8.6 Vitamin D

The term 'vitamin D' specifically refers to the parental vitamin D produced endogenously by the action of sunlight on 7-dehydrocholesterol in skin (also known as vitamin D3, or cholecalciferol), or obtained from dietary foodstuffs as either vitamin D3 or vegetable vitamin D2 (also known as ergocalciferol). Vitamin D derived from

sunlight or diet undergoes metabolism, firstly to 25-hydroxyvitamin D (25OHD) that is the main circulating form of vitamin D used to define 'vitamin D status'. At physiological concentrations, 25OHD appears to be inactive as a signalling molecule. Consequently, the target cell function of vitamin D is determined by conversion of 25OHD to active 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], which is catalysed by the vitamin D-activating enzyme 25-hydroxyvitamin D-1a-hydroxylase (CYP27B1). The 1,25$(OH)_2$D produced in this manner then functions as a steroid hormone by binding to the nuclear vitamin D receptor (VDR) and acting as a regulator of gene transcription (Hewison, 2012). Vitamin D is also commonly used to denote the lipid-soluble hormone critical for calcium homeostasis and skeletal maintenance. A precursor to the active compound is found in many plants and animal tissues and can be absorbed from the gut; it can also be derived from cell membranes in the epidermis during ultraviolet B irradiation. This compound is then hydroxylated sequentially in the liver and kidney to produce the active hormone 1,25$(OH)_2$D that binds its nuclear receptor to modulate gene expression. Recently, vitamin D hydroxylases and the nuclear receptor have been identified in many tissues, suggesting previously unrecognized roles for vitamin D. Some epidemiologic studies have also correlated low levels of the inactive storage form 25(OH)D with an increased incidence or prevalence of a variety of diseases, suggesting that large oral supplements and/or increased ultraviolet (UV) exposure might therefore improve individual health (Powers & Gilchrest, 2012).

The 25-hydroxylation of vitamin D in the liver takes place in hepatocytes and is not tightly regulated; pharmacological doses of vitamin D that are not stored in fat or muscle are readily converted to 25D. Because of difficulties in measuring plasma levels of vitamin D, assay of 25D (calcidiol) is used to provide an estimate of vitamin D status.

The normal range in the US is 2—100 nmol/L. Low plasma 25D levels indicate reduced vitamin D stores (Whyte, 1993)

Vitamin D facilitates active transport of calcium across the intestinal mucosa, partly by inducing the formation of a calcium-binding transport protein in intestinal mucosal cells (Heaney, 2005). In the absence of vitamin D, the plasma calcium tends to fall, secondary hyperparathyroidism develops and the condition known as osteomalacia/rickets arises (Nordin, Need, & Morris, 1993). This tendency to hypocalcaemia and secondary hyperthyroidism in vitamin D deficiency is not due to malabsorption of calcium but to the loss of all the bone resorbing, calcaemic action of vitamin D.

2.8.7 Calcium absorption

Calcium absorption is central to the body's calcium homeostasis (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998). Calcium nutrition not only is widely recognised as important for bone health but also has been implicated in such disorders as hypertension, preeclampsia, premenstrual syndrome, and colon cancer.

There is a great deal of variability in calcium absorption. In healthy women, gross absorption efficiency spans at least a three-fold range from 15% to 45%, even after adjustment for differences in intake (Heaney, 1999). The reasons for much of this inter-individual variability are unclear, while it is known that there is a high degree of within-individual consistency in absorptive performance over time, i.e. some individuals are efficient absorbers and others are poor absorbers (Heaney, 1999).

Blood calcium concentrations are tightly regulated and during absorption, the input levels from the intestine are compensated by downwards regulatory inputs from bone. As a result, even large ingested loads increase serum calcium in small amounts (Heaney, 1999). Under very carefully controlled conditions, the small absorptive increases that do occur can be used to compare absorbability of different sources. It does not yield absolute absorbed quantities, nor is it useful for comparing individuals or for screening or diagnosis (Heaney, 1999). Investigators have generally then used a tracer method, using stable or radioactive calcium isotope.

2.8.8 Method of calcium absorption

The investigation of calcium disorders, whether for research or clinical management purposes, requires some measurement of calcium absorption, for which three methods are available: a full calcium balance (Nordin, 1976), which requires a stay of at least 2 weeks in a metabolic unit and is impractical for routine use; a double – isotope procedure, with two stable or radioactive calcium isotopes, which is involved with technical difficulties and may require a delay of several weeks before yielding a result; and a single isotope procedure, which is the simplest, most cost effective and quickest method (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998).

It is the easiest and most accurate to use isotopic tracer methods (either stable or radioactive). Ideally the labelling of the tracer would be intrinsic, i.e. incorporated into the calcium source before its addition to the food. Such an approach is only rarely applicable to marketed (or about to be marketed) products. An alternative approach is the extrinsic labelling of the finished product, where the tracer is added to the food

product a few hours before actual consumption (Heaney, Rafferty, Dowell, & Bierman, 2005). Measuring trace element absorption from the diet requires the use of isotopes to label the trace element source.

Either radioisotopes or stable isotopes can be used for multiple labelling studies for some trace elements. Both approaches offer a number of advantages and disadvantages. Radioisotopes are less costly and easier to measure than stable isotopes, and only a small quantity of the element is required for labelling purposes, whereas stable isotopes are naturally present in the environment and doses administered must be large enough to produce a measurable change in isotope ratios. This makes the stable isotope method relatively expensive (Fairweather - Tait, Fox, Harvey, Teucher, & Dainty, 2001).

Absorption is a three-stage process comprised of uptake of the trace element from the gastrointestinal lumen by the mucosal cells, followed by intra-enterocyte transfer, and serosal transport into the systemic circulation (Fairweather - Tait, Fox, Harvey, Teucher, & Dainty, 2001). Any labelled element that is not transferred into the body but is lost through mucosal cell exfoliation is not classified as absorbed. The difference between isotope intake and excretion is called *apparent absorption*. When an allowance is made for the quantity of isotope that is lost through endogenous excretions, the term *true absorption* is used. Whole-body counting techniques can either generate apparent or true absorption data, depending on the ability to quantify endogenous excretion (Fairweather - Tait, Fox, Harvey, Teucher, & Dainty, 2001). *Fractional absorption* is a term used to describe the proportion of the dose that has been absorbed (Fairweather - Tait, Fox, Harvey, Teucher, & Dainty, 2001).

2.8.9 Extrinsic and intrinsic labelling

To measure absorption of a micronutrient such as calcium from the diet requires the use of radioactive or stable isotopes to label the trace-element source (Patterson & Veillon, 2001). Isotopic labelling of calcium in a test food will then allow differentiation between the proportion of calcium present in the test food from that derived from other sources (either dietary or endogenous in origin) and present in body fluids and tissues (Fairweather - Tait, Fox, Harvey, Teucher, & Dainty, 2001). Any isotope used to label a test food should be present in the same chemical form as the native trace element. While this can be achieved by biosynthetic (intrinsic) labelling, this method is generally expensive, impractical and time consuming (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998). An alternative strategy is extrinsic labelling where the isotope is simply mixed with the food until complete isotopic exchange occurs. Extrinsic labelling was first developed for iron using radioisotopes and is valid under conditions where complete isotopic exchange takes place prior to absorption (Fairweather - Tait, Fox, Harvey, Teucher, & Dainty, 2001)

Earlier studies on cows' milk (Nickel, Berdine, Smith, Smith, Miller, & Weaver, 1996) and calcium-containing wheat flour product (Weaver, Heaney, Martin, & Fitzsimmons, 1992) have shown that labelling of fortificant after its addition to the food can generate a similar tracer abundance to that obtained when the fortificant is labelled before it is added to the food. For foods such as cows' milk, a calcium isotope appears to be able to exchange readily with stable calcium irrespective of the physical and chemical nature of the food. In contrast, when heat treatment is not used, fortificant labelling after addition to foods such as green leafy vegetables (Heaney, Weaver, & Recker, 1988) or soymilk (Heaney, Dowell, Rafferty, & Bierman, 2000) gives rise to

non-uniform tracer distribution, and for CFSM, in particular a 50% overestimation of true absorbability (Heaney, Dowell, Rafferty, & Bierman, 2000) has been observed.

2.8.10 Single isotope radioactive test

^{45}Ca is about as close to the ideal diagnostic radionuclide as one could imagine. It has a 165-day half life which gives it a highly acceptable shelf life for the laboratory providing the test. ^{45}Ca has no gamma emission, and its weak beta particles dissipate most of its energy in the inert bone matter, with a corresponding 50-fold reduction in the possibility of biological damage (relative to a comparable radionuclide distributed in soft tissue) (Heaney, 1999). ^{45}Ca-based methods deliver a radiation dose that is a small fraction of the small fraction of the annual natural background radiation (Heaney, 1999). ^{45}Ca methods are also simple and inexpensive.

The single isotope radioactive test was introduced in 1961 by Bhandarkar *et al* (Bhandarkar, Bluhm, MacGregor, & Nordin, 1961). The researchers gave a 5 μCi ^{45}Ca in 250 mg of calcium carrier and measured the plasma radioactivity after 2 hr. The first kinetic analysis of plasma activity-time curves after the oral calcium isotope administration was undertaken in 1969 (Marshall & Nordin, 1969). Researchers gave a 5 μCi dose of radiocalcium in 20 mg of calcium carrier to fasting subjects and collected six blood samples over the following 2 hr. From the radioactive counts in these samples and by means of differential equations, they were able to calculate the rates of radiocalcium entering (α) and leaving (β) the extracellular compartment. They made three assumptions: the rate of radiocalcium entry was a function of the residual concentration in the small intestine; the rate of radiocalcium removal was a function of its concentration in the plasma, and the volume of distribution of the calcium isotope in

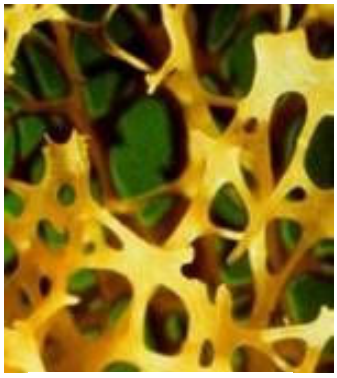
the plasma and extravascular fluid was equivalent to 15% of body weight (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998). In subsequent studies, the single isotope radioactive test yielded results that were highly correlated with calcium absorption measured by either the balance or double-isotope techniques (Marshall & Nordin, 1981; Nordin, 1976; Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998).

2.9 Osteoporosis

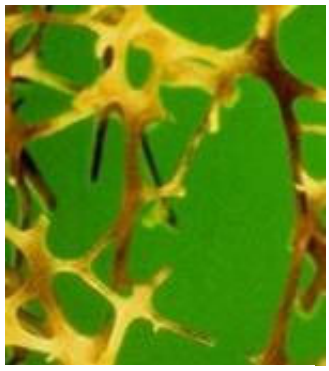
Osteoporosis is a major global health issue which is becoming increasingly important as the population ages. The World Health Organisation (WHO) estimates that 70 million people worldwide have osteoporosis. Hip fractures are the most severe consequence of osteoporosis and are associated with lengthy hospital admissions, difficulty in performing activities in daily life, nursing home placement and a high rate of mortality. The annual worldwide incidence of hip fracture is 1.5 million, a number projected to grow to 2.6 million by 2025 and to 4.5 million by 2050 (Gueldner, Grabo, Newman, & Cooper, 2008). Estimates from the Australian Bureau of Statistics 2004-2005 National Health Survey suggest that about 600,000 Australians (3% of the population) have doctor-diagnosed osteoporosis. Of these, 85% are women and 15% are men (*A picture of osteoporosis*, 2008). Osteoporosis is a condition where the bones become fragile and brittle and fracture more easily than a normal bone (Figure 2.5). Even a minor bump or fall can cause serious fracture. In Australia, half of all women and one third of men over 60 will have a fracture due to osteoporosis (*Calcium, Vitamin D and Osteoporosis - A guide for consumers*, 2008).

Osteoporosis is a skeletal disorder characterised by compromised bone strength, which predisposes the sufferer to increased risk of fractures (Camacho & Miller, 2007). To understand osteoporosis, it is important to appreciate the dynamic nature of bones. Bone is a tissue composed of a solid, thick outer layer known as cortical bone and an internal honeycomb-like structure called trabecular bone. The bone tissue is in a continual process of breakdown and rebuilding, ensuring that bones are repaired and remain strong (*A picture of osteoporosis*, 2008). Bone strength is a product of both bone density and bone quality. Bones gives shape and form to the body, support the body's weight, protect vital organs, serve as a storage area for minerals such as calcium and phosphorus, provide stem cells from bone marrow for healing and cell growth, and work in conjunction with the muscular system to assist the body with movement (Gueldner, Grabo, Newman, & Cooper, 2008). Because of its hard texture, bone is commonly thought of as an inactive tissue. However, it is a dynamic tissue in which the cells are involved in extensive interactions with one another, and with hematopoietic (blood forming) and stromal (connective tissue) cells in the bone marrow. These interactions are prominent in maintaining bone mass (Gueldner, Grabo, Newman, & Cooper, 2008).

Bone density is expressed as grams of mineral per area or volume; bone quality refers to factors such as architecture, turnover, damage accumulation and mineralisation (Camacho & Miller, 2007). Bone density can be measured by various methods but bone quality is not readily quantifiable (Camacho & Miller, 2007).



Normal Bone



Osteoporotic Bone

Image courtesy of Osteoporosis Australia

Figure 2.5 – Normal versus osteoporotic bone

(Source: Osteoporosis Australia)

2.9.1 Diagnosis of osteoporosis

Osteoporosis is most commonly diagnosed using bone densitometry. Various techniques are available to quantify bone mass such as DXA (dual-energy X-ray absorptiometry), Q-CT (quantitative computed tomography), p-DXA (peripheral dual-energy X-ray absorptiometry). The most accurate and precise technique is the central dual-energy X-ray absorptiometry (DXA) scan (Camacho & Miller, 2007).

The basic principle of the DXA scan is that two beams of X-rays are generated and allowed to pass through the area of interest, usually the spine or the hip. The density of the bone, usually determined by its calcium content, causes different attenuation of these X-ray beams. As the beams pass through the bone and soft tissues, two photoelectric peaks are quantified, and the device is able to subtract the contribution of the soft tissue to the measured density. BMD is expressed as an area measurement in grams per square centimetre (Camacho & Miller, 2007).

2.9.2 Fracture risk

Fracture risk increases significantly with age, and the incidence rises sharply after the menopausal years. Hip fractures occur about a decade later, with a sharp increase in incidence around the age of 70. A strong correlation exists between fracture risk and bone density, and this relationship is even stronger than that between cholesterol and heart disease (Figure 2.6).

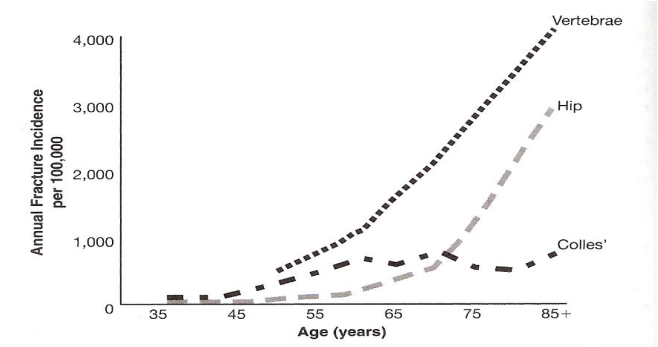


Figure 1.2. Fracture risk with aging in white women. (Adapted from Riggs BL, Melton LJ III. Involutional osteoporosis. N Engl J Med 1986;314:1676, with permission.)

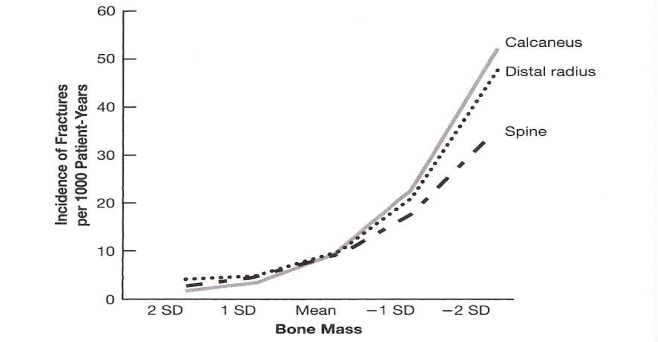


Figure 1.3. Fracture risk vs. bone density. There is an exponential relationship between decreasing bone mass and increasing incidence of fractures. (Adapted from Miller PD, Bonnick SL, Rosen CJ. Consensus of an international panel on the clinical utility of bone mass measurements in the detection of low bone mass in the adult population. Calcif Tissue Int 1996;58:207–214, with permission.)

Figure 2.6 – Relationship of fracture risk with age and bone density

(Extracted from Camacho & Miller, 2007))

2.9.3 T-Scores and Z-Scores

The T-score is the number of standard deviations that the BMD falls below or above the mean of a young healthy adult. This score is used for diagnosing osteoporosis. Z-scores compare the BMD with age and sex-matched controls and give an idea of the age appropriateness of bone loss (Camacho & Miller, 2007). The World Health Organisation (WHO) criteria are the widely accepted basis for osteoporosis diagnosis, based on the T-score. Osteoporosis is defined by a T-score equal to or less than -2.5. T-scores above this cut-off but below 1.0 define osteopenia or low bone mass. Normal BMD is 1 SD above or below the mean (T-score of -1 to + 1). An individual who has a T-score of -2.5 or less and has suffered from an osteoporotic fracture is considered to have severe or established osteoporosis (Camacho & Miller, 2007).

2.9.4 Risk factors for osteoporosis

The risk of getting osteoporosis can be reduced by paying attention to certain risk factors. Some risk factors are modifiable because changes in lifestyle or behaviours can reduce risk. The fixed risk factors are those people are born with and cannot be modified. People with fixed risk factors can substantially reduce their risk of getting osteoporosis by focusing on improving their modifiable risks factors (Table 2.2).

Table 2.2 – Risk factors for osteoporosis

(Extracted from (A picture of osteoporosis, 2008))

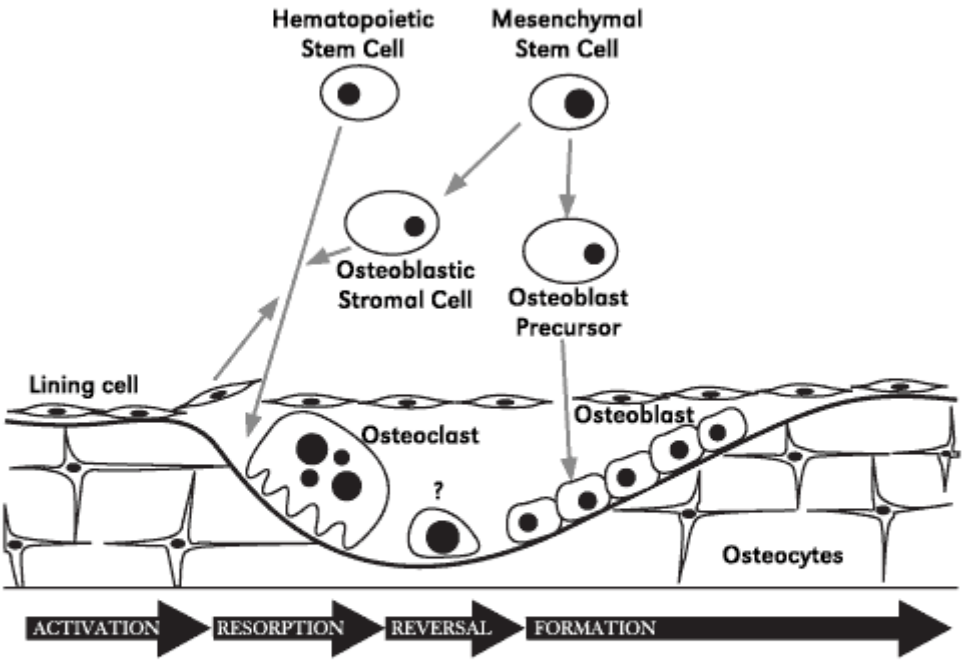
Modifiable Risk Factors	Fixed Risk factors
Physical inactivity	Family history and genetics
Low calcium and vitamin intake	Physical disability
Vitamin D deficiency	Old age
Tobacco smoking	Female sex
Excessive alcohol consumption	Previous minimal trauma fracture
Low body mass index	Other medical conditions

2.10 Bone remodelling

The skeleton is continually being renovated and replaced through the process of remodelling. This process occurs to maintain maximal bone mineral density, in addition to repairing any damage that has occurred to the bones, including micro or outright fractures. Specialised bone cells, known as osteoclasts, perform the breakdown whilst osteoblasts do the building (*A picture of osteoporosis*, 2008). Bone loss occurs when osteoblasts fail to completely refill the cavity created during resorption (Gueldner, Grabo, Newman, & Cooper, 2008). The remodelling process takes place at discrete locations near the surface of the bones just underneath the thin lining cells. The removal and replacement of bone in the remodelling cycle occurs in a carefully orchestrated sequence that involves four phases: activation, resorption, a period of reversal, and bone formation (Figure 2.7). Bone remodelling continues throughout adulthood, with each remodelling process lasting 6-9 months (Gueldner, Grabo, Newman, & Cooper, 2008). Remodelling is necessary to maintain bone strength and occurs on all bone surfaces.

Prior to adulthood, bone formation occurs at a higher rate than bone resorption, facilitating bone growth. The adult bone mass is thought to be genetically predetermined and when the adult reaches his/her late 20s to mid 30s, bone formation and resorption achieve an equal balance, so that the bone structure remains stable. Osteoporosis results from an imbalance between resorption and formation, in which bone resorption significantly exceeds formation (Gueldner, Grabo, Newman, & Cooper, 2008).

Figure 2–3. Bone Remodeling



Note: The sequence of *activation, resorption, reversal,* and *formation* is illustrated here. The activation step depends on cells of the osteoblast lineage, either on the surface of the bone or in the marrow, acting on blood cell precursors *(hematopoietic cells)* to form bone-resorbing osteoclasts. The resorption process may take place under a layer of lining cells as shown here. After a brief reversal phase, the osteoblasts begin to lay down new bone. Some of the osteoblasts remain inside the bone and are converted to osteocytes, which are connected to each other and to the surface osteoblasts. The resorption phases last only a few weeks but the formation phase is much slower, taking several months to complete, as multiple layers of new bone are formed by successive waves of osteoblasts.

Figure 2.7 – Bone remodelling

(Extracted from (Gueldner, Grabo, Newman, & Cooper, 2008))

Chapter 3

Fermentation of calcium fortified soymilk with *Lactobacillus*: effects on calcium solubility and organic acid production, and an assessment of the effect of pH on calcium fortificant solubility.*

3.1 Introduction

Soymilk is a by-product from soy bean manufacture and is increasingly included in western diets. Calcium is an essential micronutrient, obligatory for acquiring optimum bone mass (Theobald, 2005). The presence of high amounts of soluble calcium in food aids total calcium absorption (Schroder, Griffin, Specker, & Adrams, 2005; Theobald, 2005) by facilitating ionization of calcium in the digestive system. As cows' milk is high in calcium while soymilk is not naturally a rich source of this mineral, manufacturers often fortify soymilk with levels of calcium similar to those in cows' milk (~ 120 mg/100 mL) by adding calcium phosphate, calcium carbonate or calcium chloride. Not all calcium fortificants, however, are equal (Heaney, Rafferty, & Bierman, 2005), and some may have poor solubility. The bioavailability rather than the total content of calcium in soymilk is thus an important issue.

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* A version of this chapter has been published. Tang, A. L., Shah, N. P., Wilcox, G., Walker, K. Z., & Stojanovska, L. (2007). Fermentation of calcium fortified soymilk with *Lactobacillus*: Effects on calcium solubility, isoflavone conversion, and production of organic acids. *Journal of Food Science*, 72(9), 431-436.

Another potential way to improve the nutritional content of soymilk is through fermentation with probiotics (Stanton, Ross, Fitzgerald, & Van Sinderen, 2005). Probiotics are living microbial food ingredients which have been shown to have beneficial effects on human health (Isolauri, Salminen, & Ouwehand, 2004).

Probiotics help alleviate symptoms of lactose intolerance, atopic disorders; coeliac disease, and have been found useful in the treatment and prevention of diarrhoea, ulcerative colitis, irritable bowel syndrome, and urogenital tract and *Helicobacter pylori* infections (Kolida, Saulninier, & Gibson, 2006). There have also been claims for cholesterol-lowering effects (Liong & Shah, 2005), anti-carcinogenic actions (Commane, Hughes, Shortt, & Rowland, 2005) and improved immune function (Reid, 2006).

During fermentation, the growth of probiotic bacteria can lower food pH through the production of organic acids, in particular lactic and acetic acids (Tsangalis & Shah, 2004). These acids have a significant impact on the flavour profile of the fermented product.

This study investigated ways to enhance calcium bioavailability from calcium fortified soymilk through fermentation with probiotics selected for their β-glucosidase activity (Otieno, Ashton, & Shah, 2005) and production of organic acids.

3.2 Materials and methods

3.2.1 Bacteria

Pure cultures of *Lactobacillus acidophilus* ATCC4962, ATCC33200, ATCC4356, ATCC4161 and *L. casei* ASCC290 were obtained from the Victoria University Culture Collection (Werribee, Victoria, Australia). In addition, *L. plantarum*

ASCC276 was obtained from the Australian Starter Culture Research Centre Ltd (Werribee, Victoria, Australia) and *L. fermentum* VRI-003 from Probiomics Ltd (Sydney, NSW, Australia). The purity of the cultures was checked by Gram staining before organisms were stored at -80ºC in 40% glycerol.

3.2.2 Fermentation of calcium fortified soymilk

Probiotic cultures were activated through three successive transfers in MRS broth (De Man, Rogosa, & Sharpe, 1960) at 37ºC for 20 h using a 2% inoculum. Commercial calcium fortified soymilk containing 4% soy protein and 120 mg/100 mL calcium was dispersed into 500 mL bottles and heat treated at 90ºC for 30 min. Each bottle was then inoculated with 1% (v/v) inoculum of each probiotic microorganism before incubation at 37ºC for 24 h followed by storage for 14 d at 4ºC. Aliquots of 100 mL were taken aseptically from each bottle at 0 h, 12 h and 24 h of incubation; and at 7 d and 14 d of refrigerated storage for enumeration of viable bacterial populations, pH measurement and estimation of soluble calcium content. Fermentation was performed three times and measured in duplicates.

3.2.3 Enumeration of probiotic microorganisms in fermented milk

The pour plate method was used for bacterial enumeration using MRS agar. Plates were incubated at 37ºC for 3 days in an anaerobic jar (Becton-Dickinson Microbiology Systems ®, Sparks, MD, USA) with a gas generating kit ™ (Oxoid Ltd, Hampshire, UK). Populations of probiotic microorganisms are presented as $\log_{10}$ colony forming units (CFU)/mL of calcium fortified soymilk.

3.2.4 Acidification of calcium fortified soymilk to increase calcium solubility and determination of soluble calcium content

Concentrated HCL was added at different amounts to 100 mL aliquots of calcium fortified soymilk to achieve a final pH ranging from 1.0 to 7.0. After stirring, each acidified calcium fortified soymilk was allowed to stand for 10 min and then centrifuged at 14,000 × g for 30 min (Eppendorf model 5415C, Crown Scientific Pty. Ltd., Australia). Each centrifuged calcium fortified soymilk sample was then separated into 3 portions; the upper fat layer, an intermediate soluble layer and the sediment. The upper fat layer and the middle intermediate clear layer were pipetted away in order from the sediment. The intermediate layer was diluted with distilled water to standard curve range to allow for measurement of calcium content by atomic spectrophotometry (Varian SpectrAA-300/400, Palo Alto, CA, USA).

3.2.5 Sample extraction and HPLC analysis of organic acids

Organic acids including acetic, propionic, butyric, lactic and formic acids, were extracted from fermented calcium fortified soymilk (Ong, Henriksson, & Shah, 2006; Shin, Lee, Pestka, & Ustunol, 2000; Tsangalis & Shah, 2004). Briefly, 5 mL duplicate samples of homogenous calcium fortified soymilk were vortexed for 20 s with 70 μL of 15.5 N HNO_3 and 5930 μL of 0.009 N H_2SO_4. Aliquots of 1.5 mL were then transferred to 1.7 mL microtubes and centrifuged at 14,000 × g for 10 min using an Eppendorf centrifuge (Model 5415C, Crown Scientific Pty. Ltd., Australia). The supernatants were then passed through 0.23 μm Millipore filters.

The Varian HPLC system consisted of a 9012 solvent delivery system, a 9100 auto-sampler, a 9050 variable wavelength UV/VIS tunable absorbance detector and a

730 data module. Analyses were carried out with an Aminex HPX-87 H column (300 × 7.8 mm, Bio-Rad Laboratories, Richmond, CA, USA) connected to a Micro-Guard ® cartridge (Bio-Rad Laboratories) maintained at 65˚C. Sulphuric acid (0.009 N), filtered through a 0.45 μm membrane filter (Millipore, MA, USA) was used as the mobile phase at a flow rate of 0.6 mL min^{-1}. The UV detector was set at 220 nm and the samples were eluted for 20 min.

3.3 Statistical analysis

Data analysis was carried out with SPSS Inc. software (version 11.5). One-way analysis of variance (ANOVA) was used to determine significant difference between means, with significance level at $\alpha = 0.05$. Tukey's HSD test was used to perform multiple comparisons between means. All data presented are mean values of three fermentations and two replicates (n = 6), unless otherwise stated.

3.4 Results and discussion

3.4.1 Viability of the microorganisms in calcium fortified soymilk during fermentation and refrigerated storage

A minimum of 6 $\log_{10}$ CFU/mL of probiotic bacteria is required to effectively modulate intestinal microbial balance and provide a therapeutic dose (Tsangalis, Ashton, Stojanovska, Wilcox, & Shah, 2004). The aim was therefore to achieve a viable dose of at least 8 $\log_{10}$ CFU/mL during fermentation and to maintain viability above 7 $\log_{10}$ CFU/mL during refrigerated storage. Results for selected probiotic organisms are shown in Table 3.1. All cultures were commenced with a similar initial inoculum

(7 $\log_{10}$ CFU/mL). After 12 h incubation at 37˚C viability increased significantly ($P < 0.05$), with a range of viable counts from 7.90 to 9.15 $\log_{10}$ CFU/mL. After this time maximum growth had occurred in *L. casei* ASCC290. A period of 24 h fermentation is sufficient to achieve peak β-glucosidase activity and maximally transform isoflavone glucosides to aglycones (Otieno, Ashton, & Shah, 2006a; Tsangalis, Ashton, Mcgill, & Shah, 2002). After 24 h, significant increase in viability ($P < 0.05$) was seen in five strains: *L. acidophilus* ATCC33200, *L. acidophilus* ATCC4356, *L. acidophilus* ATCC4161, *L. casei* ASCC290 and *L. plantarum* ASCC276, indicating good adaptation to the calcium fortified soymilk environment and resistance to the growing accumulation of organic acids (Hughes & Hoover, 1995). At this time, viability counts for all strains were above 8.8 $\log_{10}$ CFU/mL. Seven days storage at 4˚C caused no further change in viability, and a viability $\geq$ 8.5 $\log_{10}$ CFU/mL was maintained until 14 d at 4˚C. At this time, the highest level of viability was seen with *L. acidophilus* ATCC4161 and *L. casei* ASCC290 (9.45 ± 0.23 and 9.51 ± 0.08 $\log_{10}$ CFU/mL, respectively).

Changes in pH during fermentation are shown in Table 3.2. At inoculation, the pH of calcium fortified soymilk ranged from 7.12-7.15. After 12 h fermentation with probiotic bacteria, calcium fortified soymilk exhibited a significant decrease in pH ($P < 0.05$) and this decrease was maintained at 24 h. The greatest drop in pH occurred in *L acidophilus* ATCC4161 and *L. casei*. These low pH levels were maintained during storage for 7 d and 14 d. Calcium fortified soymilk fermented with *L. casei* ASCC290 and *L. plantarum* ASCC276 exhibited the lowest pH after 14 d storage. Low pH had no adverse effect on viability even after 14 d after refrigerated storage consistent with observations by others (Donkor, Henriksson, Vasiljevic, & Shah, 2006; Tsangalis, Ashton, Stojanovska, Wilcox, & Shah, 2004).

3.4.2 Calcium solubility during fermentation

The total amount of calcium found in calcium fortified soymilk in this study was 120 mg/100 mL. At inoculation, 20-22% was in the soluble form; however the level increased during fermentation (Table 3.3). Lower pH (Table 3.2) increases calcium solubility due to the ionization of the calcium salts. After 12 h fermentation, increase in soluble calcium was seen only in *L. fermentum* VRI-003, but by 24 h there was a significant increase in calcium solubility ($P < 0.05$) across all probiotic strains. The highest levels of soluble calcium at 24 h were found in *L. acidophilus* ATCC 4962, *L. casei* ASCC290, *L. plantarum* ASCC276 and *L. fermentum* VRI-003. Further significant ($P < 0.05$) increase in calcium solubility occurred during 7 d and 14 d refrigerated storage. *L. acidophilus* ATCC4356 and *L. acidophilus* ATCC4161 expressed the least increase in calcium solubility, while *L. acidophilus* ATCC4962 and *L. casei* ASCC290 expressed a 79% and 77% increase in calcium solubility respectively, after 14 d storage. *L. casei* ASCC290 also had the lowest pH (Table 3.2) at this time. Improved calcium solubility in fermented calcium fortified soymilk (CFSM) is a useful property with the potential to facilitate calcium absorption *in vivo* (Theobald, 2005).

3.4.3 Effect of pH on calcium solubility

From knowledge of calcium chemistry, it was assumed that decreased pH during fermentation increases calcium solubility in calcium fortified soymilk. To test this directly, calcium solubility was measured directly after addition of acid to calcium fortified soymilk to alter pH (Figure 3.1). The pH of unfermented calcium fortified soymilk is 7.0-7.2 and at this pH the percentage soluble calcium was 12.3 ± 0.6% (mean

± SD). Calcium solubility gradually increased as pH decreased (Figure 3.1). The most significant increase of 69.7 ± 1.1% ($P < 0.05$) occurred at pH 4.0. This is the pH that was observed after 24 h fermentation of calcium fortified soymilk with probiotic strains. Greatest calcium solubility occurred at pH 1.0 resulting in a solubility of 93.8 ± 5.5%. These data confirm our hypothesis that acid production during fermentation of calcium fortified soymilk was one of the major contributing factors to calcium solubility.

3.4.4 Production of organic acids

The production of L (+)-lactic and acetic acids after fermentation and storage of the fermented calcium fortified soymilk is shown in Table 3.4 Propionic, formic and butyric acid could not be detected in any of the samples. After 12 h fermentation, the levels of lactic and acetic acids increased in all samples. Greatest accumulation of lactic acid at 12 h was seen in calcium fortified soymilk fermented with *L. acidophilus* ATCC4962 and *L. plantarum* ASCC276, while *L. acidophilus* ATCC4356 and *L. casei* ASCC290 produced the most acetic acid. Concentrations of lactic and acetic acid continued to increase after 24 h fermentation, with *L. acidophilus* ATCC4962 producing the most lactic acid (243.3 ± 1.0 mg/100 mL) and *L. acidophilus* ATCC33200 producing the most acetic acid (45.6 ± 0.5 mg/100 mL). As acetic acid contributes a vinegary taste to fermented products, it is desirable to keep acetic acid levels low (Tsangalis & Shah, 2004). During storage, lactic acid and acetic acid production continued to increase as reflected by a decrease in pH (Table 3.2).

3.5 Conclusions

Viability of probiotic bacteria was maintained above 7 $\log_{10}$ CFU/mL after fermentation and storage of calcium fortified soymilk. The significant increase in calcium solubility observed in calcium fortified soymilk after fermentation was related to lowered pH associated with production of lactic and acetic acids. Fermentation of calcium fortified soymilk with selected probiotics might therefore be a promising way to enhance calcium bioavailability from calcium fortified soymilk increasing its potential to improve bone health.

Table 3.1 – Viable microbial count (log_{10} CFU/mL) of calcium fortified soymilk fermented with selected probiotic microorganisms for 12 h and 24 h at 37˚C and stored for 7 d and 14 d at 4˚C

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
0 h	7.49 ± 0.18^{a}	7.48 ± 0.07^{a}	7.39 ± 0.37^{a}	7.48 ± 0.15^{a}	7.54 ± 0.22^{a}	7.31 ± 0.05^{a}	7.32 ± 0.25^{a}
12 h	8.35 ± 0.50^{b}	8.60 ± 0.31^{b}	8.16 ± 0.26^{b}	8.84 ± 0.34^{bc}	9.15 ± 0.06^{b}	8.52 ± 0.22^{b}	7.90 ± 0.13^{ab}
24 h	8.93 ± 0.52^{b}	9.10 ± 0.26^{c}	8.66 ± 0.19^{bc}	9.42 ± 0.33^{c}	9.65 ± 0.45^{bc}	8.87 ± 0.07^{c}	8.98 ± 0.77^{b}
7 d	8.96 ± 0.42^{b}	9.16 ± 0.36^{c}	8.95 ± 0.64^{bc}	9.35 ± 0.19^{c}	9.49 ± 0.08^{bc}	8.98 ± 0.13^{c}	8.53 ± 0.31^{b}
14 d	9.00 ± 0.48^{b}	9.35 ± 0.25^{c}	8.74 ± 0.24^{c}	9.45 ± 0.23^{c}	9.51 ± 0.08^{c}	8.86 ± 0.08^{c}	8.58 ± 0.47^{c}

Results expressed as means ± standard error (n = 6). Means with different lowercase letters are significantly different ($P < 0.05$)

Table 3.2 – pH of calcium fortified soymilk fermented with selected probiotic microorganisms for 12 h and 24 h at 37˚C and stored for 7 d and 14 d at 4˚C

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
0 h	7.15 ± 0.04^{a}	7.16 ± 0.03^{a}	7.12 ± 0.06^{a}	7.14 ± 0.06^{a}	7.15 ± 0.06^{a}	7.15 ± 0.06^{a}	7.16 ± 0.05^{a}
12 h	6.11 ± 0.15^{ab}	5.96 ± 0.33^{b}	6.23 ± 0.36^{b}	5.86 ± 0.14^{b}	5.72 ± 0.37^{b}	5.86 ± 0.24^{b}	6.13 ± 0.10^{b}
24 h	5.42 ± 0.53^{b}	5.10 ± 0.29^{c}	5.78 ± 0.26^{b}	4.91 ± 0.30^{c}	4.78 ± 0.06^{c}	4.98 ± 0.21^{c}	5.57 ± 0.26^{bc}
7 d	5.32 ± 0.52^{b}	5.00 ± 0.28^{c}	5.77 ± 0.13^{b}	5.14 ± 0.42^{bc}	4.64 ± 0.05^{c}	4.88 ± 0.15^{c}	5.51 ± 0.31^{bc}
14 d	5.22 ± 0.48^{b}	4.92 ± 0.32^{c}	5.68 ± 0.20^{b}	4.72 ± 0.37^{c}	4.55 ± 0.06^{c}	4.76 ± 0.16^{c}	5.40 ± 0.38^{c}

Results are mean ± standard error (n = 6). Means with different lowercase letters are significantly different ($P < 0.05$).

Table 3.3 – Percentage soluble (%) calcium in calcium fortified soymilk fermented with selected probiotic microorganisms for 12 h and 24 h at 37˚C and stored for 7 d and 14 d at 4˚C

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
0 h	20.84 ± 1.99^{aA}	23.49 ± 3.19^{aA}	22.17 ± 1.12^{aA}	22.70 ± 0.51^{aA}	22.91 ± 0.65^{aA}	20.49 ± 2.46^{aA}	23.11 ± 0.15^{aA}
12 h	20.80 ± 0.89^{aB}	18.28 ± 0.28^{aA}	19.74 ± 0.20^{aAB}	19.54 ± 0.70^{aAB}	20.58 ± 0.60^{aB}	23.43 ± 0.81^{aC}	32.27 ± 0.88^{bD}
24 h	89.25 ± 0.79^{bD}	42.83 ± 0.78^{bB}	35.15 ± 0.66^{bA}	38.04 ± 0.37^{bA}	87.00 ± 0.94^{bD}	82.58 ± 1.35^{bC}	82.39 ± 2.30^{cC}
7 d	91.67 ± 4.41^{cB}	72.74 ± 6.28^{cB}	55.61 ± 2.13^{cA}	64.80 ± 1.44^{cA}	93.33 ± 1.50^{cB}	83.33 ± 1.66^{cB}	83.33 ± 0.41^{dB}
14 d	99.49 ± 3.30^{dCD}	92.19 ± 3.72^{dBC}	71.58 ± 4.16^{dA}	81.33 ± 1.36^{dA}	100.00 ± 1.13^{dD}	95.86 ± 5.36^{dBCD}	87.65 ± 0.73^{eB}

Results are mean ± standard error (n = 6). Means in the same column with different lowercase letters and means in the same row with different capital letters are significantly different ($P < 0.05$).

Table 3.4 – Concentration of L(+)-lactic acid and acetic acid (mg per 100 mL) in calcium fortified soymilk fermented with selected probiotic microorganisms for 12 h and 24 h at 37˚C and stored for 7 d and 14 d at 4˚C

	L. acidophilus ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
L(+)-Lactic acid							
0 h	4.4 ± 0.3^{aD}	3.3 ± 1.1^{aC}	1.3 ± 0.7^{aA}	2.1 ± 0.2^{aAB}	3.8 ± 0.4^{aCD}	3.0 ± 0.2^{aBC}	2.9 ± 0.4^{aBC}
12 h	69.3 ± 1.2^{bE}	17.8 ± 0.7^{bA}	20.8 ± 0.8^{aAB}	22.9 ± 2.6^{aB}	45.8 ± 0.8^{bC}	75.3 ± 3.6^{bF}	30.0 ± 1.2^{bD}
24 h	243.3 ± 1.0^{cF}	67.2 ± 0.9^{cB}	39.1 ± 6.7^{aA}	80.4 ± 0.5^{bC}	165.2 ± 1.0^{cD}	107.7 ± 0.9^{cG}	57.7 ± 1.6^{cE}
7 d	277.0 ± 30.6^{dC}	154.6 ± 1.3^{dB}	$93.2 \pm 0.3b^{A}$	101.0 ± 11.4^{bA}	169.9 ± 1.2^{cB}	128.9 ± 49.2^{dA}	174.0 ± 1.2^{dB}
14 d	337.9 ± 4.5^{eB}	177.6 ± 7.2^{eA}	138.9 ± 5.4^{cA}	134.2 ± 25.1^{cA}	314.7 ± 8.6^{dB}	254.4 ± 1.0^{eA}	319.8 ± 10.0^{eB}
Acetic acid							
0 h	2.0 ± 3.1^{aA}	0.5 ± 1.3^{aA}	ND	7.2 ± 0.7^{aB}	ND	ND	ND
12 h	15.4 ± 1.3^{bC}	12.2 ± 0.6^{bB}	41.4 ± 1.8^{bE}	11.8 ± 0.5^{abB}	30.4 ± 2.8^{bcD}	9.9 ± 1.2^{bB}	5.0 ± 0.8^{bA}
24 h	26.3 ± 1.5^{cC}	45.6 ± 0.5^{cE}	32.6 ± 6.2^{cD}	15.2 ± 1.5^{bB}	30.5 ± 1.9^{bcD}	15.3 ± 0.6^{cB}	9.8 ± 1.3^{cA}
7 d	33.1 ± 1.2^{dB}	49.9 ± 2.0^{cD}	49.5 ± 0.7^{dD}	41.5 ± 8.3^{cC}	32.1 ± 1.0^{cB}	12.0 ± 0.5^{dA}	47.8 ± 1.0^{dD}
14 d	42.8 ± 6.4^{eBC}	56.3 ± 7.9^{dD}	54.3 ± 7.6^{dD}	$51.6 \pm 0.9d^{CD}$	28.1 ± 0.3^{bA}	30.9 ± 1.7^{eA}	42.1 ± 2.9^{eB}

Results are mean ± standard error (n = 6). Means in the same column with different lowercase letters and means in the same row with different capital letters are significantly different ($P < 0.05$). ND = not detected

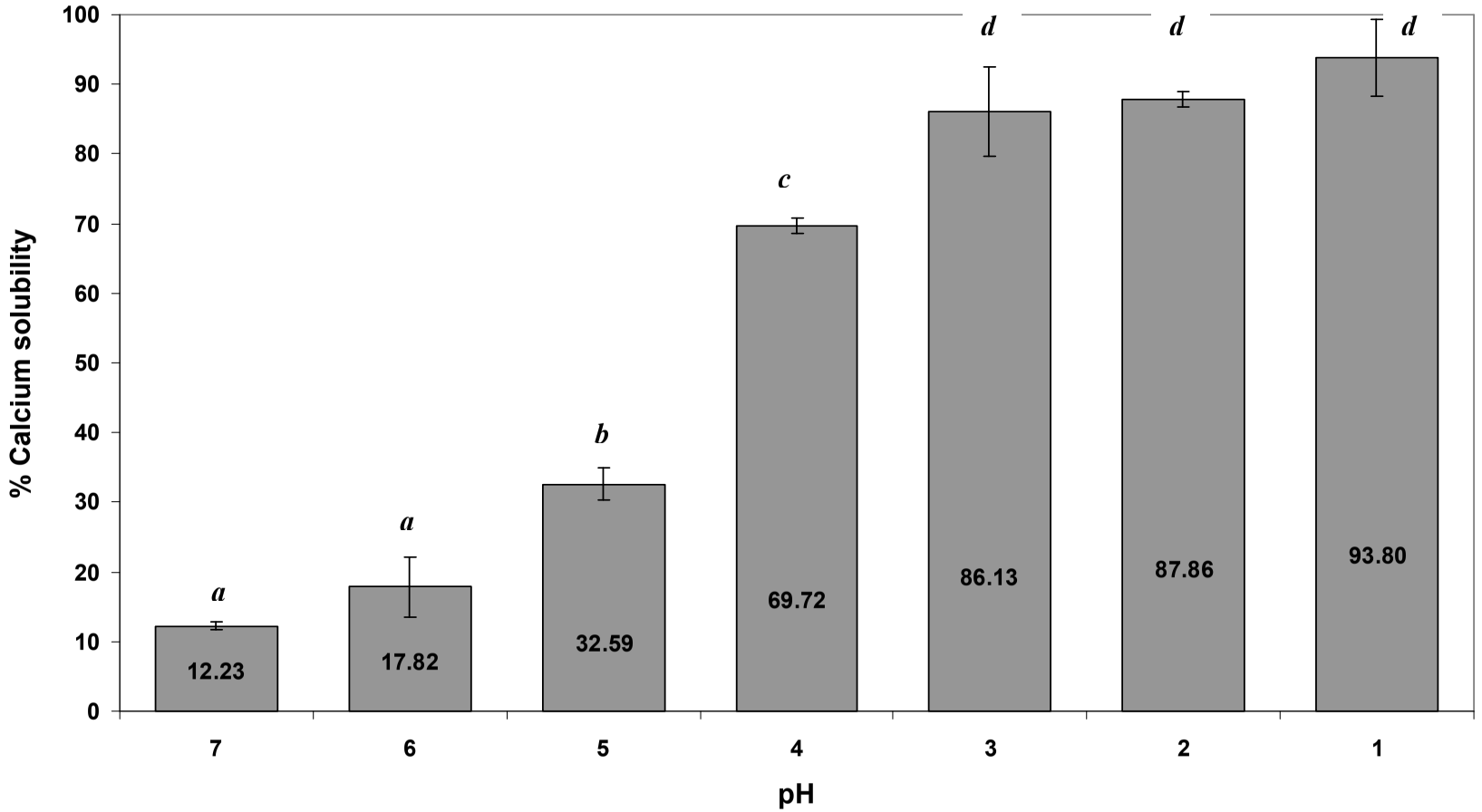

Figure 3.1 – Effect of pH on percentage calcium solubility in calcium fortified soymilk

Means in the same column with different lowercase letters are significantly different ($P < 0.05$)

Chapter 4
Production of biologically active soy isoflavones during the fermentation of calcium fortified soymilk with Lactobacillus.†

4.1 Introduction

Isoflavones are phytoestrogens. They have a structural as well as functional similarity to the human hormone oestrogen, and have frequently been consumed to alleviate menopausal symptoms associated with decline in oestrogen levels. Of all plants containing phytoestrogens, soy isoflavones are the most studied (Brouns, 2002). Researchers have recognised that Asian women consuming a diet rich in soy have better cardiovascular and bone health than western women (Ho, Chan, So, Yi, & Sham, 2009). Soy is rich in isoflavones and these compounds are related to the group of nutrients called flavonoids that are found in fruits and vegetables. There has been some scientific evidence that isoflavones may help cardiovascular health and improve bone architecture (Setchell & Cassidy, 1999b).

Isoflavones in soymilk may also bring about many benefits to peri- and post menopausal women, including: relief from hot flushes (Jefferson, 2005); improved lipid

† A version of this chapter has been published. Tang, A. L., Shah, N. P., Wilcox, G., Walker, K. Z., & Stojanovska, L. (2007). Fermentation of calcium fortified soymilk with *Lactobacillus*: Effects on calcium solubility, isoflavone conversion, and production of organic acids. *Journal of Food Science*, 72(9), 431-436.

profiles and protection against oxidative damage to DNA (Ryan-Borchers, Soon Park, Chew, McGuire, Fournier, & Beerman, 2006a); and maintenance of bone health (Atkinson, Compston, Day, Dowsett, & Bingham, 2004; Lydeking-Olsen, Beck-Jensen, Setchell, & Holm-Jensen, 2004; Setchell & Lydeking-Olsen, 2003b).

One of the most important factors affecting the functional role of the isoflavones in the human body is whether these compounds are actually absorbed from the gastrointestinal tract (Brouns, 2002). Most phytoestrogens are bound to carbohydrates known as glycones. Only a very small portion of the aglycones appear free of carbohydrate. Over 90% of the isoflavones in soymilk exist as glucosidic forms (Tsangalis, Ashton, Stojanovska, Wilcox, & Shah, 2004), yet it is the aglycone forms of phytoestrogen that are the most bioavailable.

Fermentation of soymilk with β-glucosidase-producing probiotic strains, allows the acetyl- and β-glucoside isoflavones to undergo enzymatic hydrolysis into aglycone structures (Tsangalis, Ashton, Stojanovska, Wilcox, & Shah, 2004). Aglycones are absorbed faster and in greater amounts than their corresponding glucosides (Izumi, Piskula, Osawa, Obata, Tobe, Saito, Kataoka, Kubota, & Kikuchi, 2000; Kano, Takayanagi, Harada, Sawada, & Ishikawa, 2006), and are also a more potent bioactive.

The isoflavones found in soybeans include genistein, daidzein and glycetein. These are an isomeric class of flavonoids with aglycone structures similar in form to human oestrogen, that may mimic the functions of oestradiol in the human body (Setchell & Cassidy, 1999a). Studies indicate that consumption of isoflavones can have bone-sparing effects over the long term (Setchell & Lydeking-Olsen, 2003b) not only by attenuating bone loss (Atkinson, Compston, Day, Dowsett, & Bingham, 2004; Chen, Ho, Lam, Ho, & Woo, 2003b) but also by enhancing calcium absorption (Zafar, Weaver, Jones, Moore, & Barnes, 2004).

This study investigated the biotransformation of isoflavones to their aglycone forms in calcium fortified soymilk before and following fermentation with probiotics selected for their β-glucosidase activity.

4.2 Materials and methods

4.2.1 Bacteria

Pure cultures of *Lactobacillus* spp. (LAB) including; *Lactobacillus acidophilus* ATCC4962, ATCC33200, ATCC4356, ATCC4161 and *L. casei* ASCC290 were obtained from the Victoria University Culture Collection (Werribee, Victoria, Australia). In addition, *L. plantarum* ASCC276 was obtained from the Australian Starter Culture Research Centre Ltd (Werribee, Victoria, Australia) and *L. fermentum* VRI-003 from Probiomics Ltd (Sydney, NSW, Australia). The purity of cultures was checked by Gram staining before organisms were stored at -80ºC in 40% glycerol.

4.2.2 Fermentation of calcium fortified soymilk

Probiotic cultures were activated through three successive transfers in MRS broth (De Man, Rogosa, & Sharpe, 1960) at 37ºC for 20 h using a 2% inoculum. Commercial calcium fortified soymilk containing 4% soy protein and 120 mg/100 mL calcium was dispersed into 500 mL bottles and heat treated at 90ºC for 30 min. Each bottle was then inoculated with 1% (v/v) inoculum of each probiotic microorganism before incubation at 37ºC for 24 h followed by storage for 14 d at 4ºC. Aliquots of 100 mL were taken aseptically from each bottle at 0 h, 12 h and 24 h of incubation, and at 7 d and 14 d of refrigerated storage for enumeration of viable bacterial populations,

pH measurement and estimation of soluble calcium content. Fermentation was performed three times and measured in duplicates.

4.2.3 Isoflavone standards and HPLC reagents

Genistein, daidzein and glycitein were purchased from Sigma Chemical Co.(Missouri, USA). 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, glycetein, daidzein, and equilenin were prepared in HPLC grade methanol, and daidzin, genistin and glycitin 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. Standards were prepared using HPLC grade methanol (Labscan Analytical Sciences, Bangkok, Thailand) and trifluoro-acetic acid and ammonium acetate from Sigma. All reagents used were filtered through a 0.5-μm membrane (Millipore®, Bedford, MA, USA) (Tsangalis, Ashton, Mcgill, & Shah, 2002).

4.2.4 Extraction and HPLC analysis of isoflavones

The extraction of isoflavone aglycone and glucoside isomers from 1.00 g of freeze-dried calcium fortified soymilk was performed in duplicate as described by Setchell and co-workers (Setchell, Brown, Desai, Zimmer-Nechemias, Wolfe, Brashear, Kirschner, Cassidy, & Heubi, 2001a) with some modifications as stated below. High

performance liquid chromatography isocratic elution was used to isolate isoflavones for detection. The mobile phase consisted of 100% methanol and 10 mM ammonium acetate buffer (50:50) containing 1 mL of trifluoro-acetic acid per litre of solvent mixture. The flow rate was set at 0.95 mL/min. An injection volume of 25 μL was set for standards and samples with a run time of 40 min and a UV detector at 260 nm was used (Otieno, Ashton, & Shah, 2006c). Isoflavone concentrations were calculated as milligrams isoflavones/100 mL soymilk.

4.2.5 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 × 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).

4.3 Statistical analysis

Data analysis was carried out with SPSS Inc. software (version 11.5). One-way analysis of variance (ANOVA) was used to determine a significant difference between

means, with significance level at $\alpha = 0.05$. Tukey's HSD test was used to perform multiple comparisons between means. All data presented are mean values of three fermentations and two replicates (n = 6), unless otherwise stated.

4.4 Results and discussion

4.4.1 Enzymic hydrolysis of glucosides into aglycones in fermented calcium fortified soymilk

The hydrolysis of glucoside isoflavones to aglycones during calcium fortified soymilk fermentation is reported to be due to the action of β-glucosidase (Chien, Huang, & Chou, 2006; Tsangalis, Ashton, Mcgill, & Shah, 2002). Figure 4.1 displays structural representations of isoflavone isomers and the transformations caused by bacterial-induced enzymic hydrolysis. Isoflavone aglycones including daidzein, glycetein and genistein were measured in calcium fortified soymilk fermented with seven probiotic strains. In all seven cases, significant bioconversion ($P < 0.05$) of glucosides into aglycone isoflavones was observed (Tables 4.1, 4.2 and 4.3).

4.4.2 Concentration of daidzein in fermented calcium fortified soymilk

Table 4.1 shows the concentration of isoflavones aglycones in calcium fortified soymilk (mg per 100 mL) fermented with seven strains of LAB. All strains had a significant increase in daidzein after 24 h incubation and this concentration was maintained throughout storage. Diadzein content of CFSM fermented with *L. acidophilus* ATCC4962 peaked at 7 d storage but decreased at 14 d storage. A slight decrease in daidzein concentration was observed between 7 d to 14 d storage in most

strains, except for *L. casei* ASCC290 and *L. Fermentum* VRI-003, which demonstrated a consistent increase throughout. *L. acidophilus* ATCC4161 had the highest bioconversion of daidzein peaking at 7 d storage at 4°C.

4.4.3 Concentration of glycetein in fermented calcium fortified soymilk

A significant increase in glycetein was observed as early as after 12 h incubation in each CFSM incubated with individual strains of *Lactobacillus* spp. (Table 4.2). The glycetein content increased significantly again at 24 h incubation, and this increase peaked in most strain of *Lactobacillus* spp. at 7 d storage at 4°C except for *L. casei* ASCC290, *L. plantarum* ASCC276 and *L. fermentum* VRI-003 where the glycetein content peaked at 24 h. All strains had similar glycetein content after 24 h incubation at 37°C. *L acidophilus* ATCC4356 had the highest content at 7 d and *L. fermentum* VRI-003 had the highest content after 14 d storage, displaying the stability potential of glycetein in this case.

4.4.4 Concentration of genistein in fermented calcium fortified soymilk

The concentration of genistein was highest of all aglycones (Table 4.3). All seven strains of LAB exhibited similar high rates of bioconversion of glycones to aglycones during fermentation in CFSM. Genistein content had increased significantly after 12 h incubation at 37°C and concentrations continued to increase over 24 h. Maximal levels were observed during fermentation of *L. acidophilus* ATCC4962 in soymilk for 12 h, while L. *plantarum* ASCC276 exhibited the lowest level of bioconversion. *L. acidophilus* ATCC33200 displayed the highest genistein concentration after 7 d storage at 4°C, although this level then decreased over the

following seven days. *L. fermentum* VRI-003 had the highest levels of daidzein, glycetein and genistein after 24 h fermentation with mean (± SD) of 2.74 ± 0.16, 3.92 ± 0.21 and 10.60 mg/100 mL respectively.

Figures 4.2, 4.3 and 4.4 show aglycone concentration after fermentation and storage in *L. acidophilus* ATCC4962, *L. acidophilus* ATCC33200 and *L. casei* ASCC290 respectively. At 0 h, CFSM with *L. acidophilus* ATCC4962 (Figure 4.2) had mean concentrations of daidzein, glycetein and genistein at 0.18 ± 0.36, 0.22 ± 0.48 and 0.79 ± 1.03 mg/100 mL respectively. Diadzein concentration did not increase significantly until after 24 h, when levels reached 1.25 ± 0.79 mg/100 mL. Diadzein thereafter continued to rise, peaking after 7 d storage at 2.51 ± 0.98 mg/100 mL, indicating that the hydrolysis of glucosides continued during storage. Although daidzein is regarded as the most stable of the isoflavone aglycones (Otieno, Ashton, & Shah, 2007), as storage extended to 14 d, a significant decrease ($P < 0.05$) in daidzein concentration was observed. Concentrations of both glycetein and genistein had also increased significantly ($P < 0.05$) by 12 h, remaining almost constant at 24 h. Further non significant changes ($P > 0.05$) during 7 d and 14 d storage were observed. Similar bioconversions were apparent in *L. acidophilus* ATCC33200 and *L. casei* ASCC290 (Figures 4.3 and 4.4).

4.4.5 Discussions

The overall increase in level of aglycone concentration after fermentation and storage is highly significant with genistein achieving the highest concentration overall. Degradation of aglycones in soymilk has been attributed to storage temperature and hydrolysis by microbial enzymes (Chung, Kim, Lee, Cho, Kwon, Park, Jeong, Kim, &

Kim, 2007; Otieno, Ashton, & Shah, 2007). β-glucosidase activity was not measured during the biological actives aglycone measurement, the enzyme is known to be stable at the pH ranging from 2.2 to 7.5, and its maximum activity was obtained at pH 3.6. The enzymatic activity gradually increased in the range from 40 to 60°C, but a sharp decrease occurred at 65°C (Ma, Leng, Xu, Zhu, Shi, Tao, Chen, Long, & Chen, 2011). In this study, even at a slow activity, the enzyme seems to be still active at 4°C observed by the increase in aglycone content at 7 d storage at 4°C. Biological active aglycones are very stable at high temperatures and although the conjugation profile can be influenced by heat, the total isoflavone concentration in the soy protein remains constant (Setchell, 1998).

Many studies in the literature have examined the role and efficacy of soy isoflavones on bone health (Okabe & Tanimoto, 2008; Song, Paik, & Juong, 2008) and the importance of these compounds has been recognized in those reviews (Ho, Chan, Yi, Wong, & Leung, 2001; Setchell & Lydeking-Olsen, 2003a). Although some findings have been inconclusive (Weaver & Cheong, 2005), findings both in rat models (Fonseca & Ward, 2004; Mathey et al., 2007) and in humans (Lydeking-Olsen, Beck-Jensen, Setchell, & Holm-Jensen, 2004) on the effect of aglycone isoflavones on bone health have been promising. Further long term studies are needed however. One problem encountered in longer human studies is the question of the degree of compliance to prescriptions for soy foods (Setchell & Cassidy, 1999b). Significant increases in bone mineral content have been reported in post menopausal women consuming isolated soy protein over a 6 months period compared with a diet containing casein (Potter, Baum, Teng, Stillman, & Erdman, 1998). Another study examined the biochemical markers of bone turn over in 17 post menopausal women. This study showed a reduction in urinary excretion of chemical blood markers for bone loss,

including a 10% reduction in D-pyrodinoline ($P < 0.05$) and a 24% reduction in N-telopeptide ($P < 0.001$) after 3 months on a diet of 60 g isolated soy protein (Setchell & Cassidy, 1999b).

Other factors can impact on the effects of soy protein on bone health. While an intake of genistein and daidzein by ovariectomised rats provided bone sparing effects, the addition of indigestible sugars, such as short chain fructooligosaccharides or live microbacteria such as *L. casei*, to the diet of these animals was shown to significantly improve the protective effects of daidzein on the skeleton (Mathey et al., 2007). In humans and in rats, aglycone forms of isoflavones (daidzein and genistein) are degraded by the gut microflora, present and supplied by the fermentation process, to a series of metabolic products, in particular equol which is a bacterial degradation product of daidzein. Diadzein has been reported to be more bioavailable than genistein in humans (Xu, Wang, Murphy, Cook, & Hendrich, 1994). It has been suggested that the higher bone sparing activity provided by daidzein may be explained by the conversion of daidzein into equol, the major phenolic compound found in urine, blood and bile of rats maintained on an isoflavone diet (Mathey et al., 2007). Diadzein when combined with a high calcium diet, had a favourable affect on cortical and trabecular bone as indicated by increased femur and lumbar vertebrae BMD and biomechanical strength (Fonseca & Ward, 2004). Another study conducted on rats reported that ^{45}Ca absorption was significantly higher ($P < 0.01$) when isoflavones were added to the diet and concluded that isoflavones may enhance calcium absorption (Zafar, Weaver, Jones, Moore, & Barnes, 2004). Another study by Ho *et al.*, 2001 showed that soy intake had a significant effect on the maintenance of spinal bone mineral density in women aged 30-40 years (Ho, Chan, Yi, Wong, & Leung, 2001). Also the incidence of osteoporosis-related fractures is lower in Asian population, where soy consumption is higher

compared to consumption in the western communities (Ho, Chan, Yi, Wong, & Leung, 2001).

In our study, *L. acidophilus* ATCC4962, *L. acidophilus* ATCC33200 and *L. casei* ASCC290 (Figure 4.2, 4.3 and 4.4) showed the highest bioconversion; though all of the strains showed significant bioconversion levels in the CFSM. Fermentation of the CFSM increases the bioconversion of isoflavone into bioavailable aglycones. These aglycones may enhance the calcium bioavailability of the calcium fortificant through various mechanisms and improve bone health in post menopausal women who are at most risk of osteoporosis.

4.5 Conclusions

All of the seven strains of *Lactobacillus* spp. showed good growth and acid production in CFSM as demonstrated in Chapter 3. Hydrolysis of the glycoside moiety depends on various factors such as the incubation conditions and the β-glucosidase activity of the *Lactobacillus* strain. In this study, the seven strains were selected for their known β-glucosidase activities. These strains had never previously been fermented in this brand of commercial calcium fortified soymilk. This study shows that there was a significant increase in the concentrations of all the bioavailable aglycones including glycetein, daidzein and genistein after fermentation. *L. fermentum* VRI-003 had the highest levels of daidzein, glycetein and genistein at 24 h fermentation, and all the other strains also had similar bioconversion levels after 24 h. A calcium fortification system together with the bioconversion of soy isoflavones into bioavailable aglycones in the probiotic soymilk may enhance calcium bioavailability, bone health, as well as provide the known health benefits from probiotic consumption.

Table 4.1 – Concentration of daidzein **in CFSM (mg per 100mL) fermented by the seven strains of LAB at 0, 12, 24 h incubation at 37°C and 7 and 14 d storage at 4°C**

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
0 h	0.18 ± 0.36^{aB}	ND	ND	ND	ND	ND	0.30 ± 0.37^{aB}
12 h	0.20 ± 0.13^{aA}	0.64 ± 0.70^{bB}	ND	1.91 ± 0.31^{bcA}	ND	ND	ND
24 h	1.25 ± 0.79^{bA}	2.16 ± 0.40^{cA}	2.14 ± 0.18^{bA}	2.40 ± 0.28^{bcA}	2.05 ± 0.29^{bA}	1.58 ± 0.21^{bA}	2.74 ± 0.16^{bA}
7 d	2.51 ± 0.98^{cABC}	2.76 ± 0.25^{cABC}	2.92 ± 0.37^{cBC}	3.07 ± 0.48^{dC}	1.97 ± 0.18^{bA}	2.13 ± 0.15^{cAB}	2.59 ± 0.49^{bABC}
14 d	1.75 ± 0.34^{bcA}	2.29 ± 0.47^{cAB}	1.80 ± 0.62^{bA}	2.59 ± 0.46^{cdB}	2.10 ± 0.21^{bAB}	2.08 ± 0.42^{cAB}	2.73 ± 0.45^{bB}

Results are mean ± standard deviation (n = 8). Means in the same column with different lowercase letters and means in the same row with different capital letters are significantly different ($P < 0.05$).

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

Table 4.2 – Concentration of glycetein in CFSM (mg per 100mL) fermented by the seven strains of LAB at 0, 12, 24 h incubation at 37°C and 7 and 14 d storage at 4°C

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
0 h	0.22 ± 0.48^{aA}	0.44 ± 0.19^{aA}	0.17 ± 0.27^{aA}	0.52 ± 0.08^{aA}	0.22 ± 0.40^{aA}	ND	1.18 ± 0.68^{bB}
12 h	3.51 ± 0.08^{bcB}	2.00 ± 1.28^{bB}	3.19 ± 0.26^{bB}	3.17 ± 0.33^{bB}	2.57 ± 1.62^{bB}	2.18 ± 0.15^{bB}	ND
24 h	3.58 ± 0.43^{bcA}	3.80 ± 0.30^{cA}	3.62 ± 0.21^{cA}	3.74 ± 0.36^{cdA}	3.75 ± 0.10^{cA}	3.82 ± 0.47^{cA}	3.92 ± 0.21^{cA}
7 d	3.99 ± 0.40^{cC}	4.06 ± 0.25^{cC}	4.11 ± 0.39^{dC}	4.02 ± 0.22^{dC}	2.99 ± 0.17^{bcA}	3.38 ± 0.63^{cAB}	3.65 ± 0.24^{cCD}
14 d	3.22 ± 0.55^{bAB}	3.17 ± 0.31^{cAB}	2.93 ± 0.14^{bA}	3.43 ± 0.24^{bcABC}	3.28 ± 0.63^{bcABC}	3.70 ± 0.46^{cBC}	3.97 ± 0.44^{cC}

Results are mean ± standard deviation (n = 8). Means in the same column with different lowercase letters and means in the same row with different capital letters are significantly different ($P < 0.05$).

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

Table 4.3 – Concentration of genistein in CFSM (mg per 100mL) fermented by the seven strains of LAB at 0, 12, 24 h incubation at 37°C and 7 and 14 d storage at 4°C

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
0 h	0.79 ± 1.03^{aA}	0.97 ± 0.24^{aA}	1.08 ± 0.54^{aA}	ND	0.43 ± 0.80^{aA}	ND	4.03 ± 1.88^{bB}
12 h	10.24 ± 0.50^{bC}	6.76 ± 2.62^{bB}	9.13 ± 0.50^{bcBC}	8.79 ± 0.28^{bBC}	8.33 ± 2.62^{bBC}	2.99 ± 1.50^{bA}	1.00 ± 0.00^{aA}
24 h	10.05 ± 0.93^{bAB}	10.70 ± 0.92^{cdCD}	10.10 ± 0.40^{aABC}	10.64 ± 0.60^{cdABC}	11.43 ± 1.21^{cC}	9.31 ± 0.69^{cA}	10.60 ± 0.48^{cABC}
7 d	11.10 ± 0.96^{bBC}	12.20 ± 0.86^{dC}	11.97 ± 1.17^{dC}	11.08 ± 0.99^{dBC}	9.74 ± 0.83^{bcAB}	8.91 ± 1.93^{cA}	9.95 ± 0.15^{cAB}
14 d	10.03 ± 0.99^{bABC}	9.58 ± 0.59^{cAB}	8.74 ± 0.54^{bA}	10.00 ± 0.56^{cABC}	10.04 ± 1.07^{bcBC}	9.58 ± 0.79^{cAB}	11.04 ± 0.78^{cC}

Results are mean ± standard deviation (n = 8). Means in the same column with different lowercase letters and means in the same row with different capital letters are significantly different ($P < 0.05$).

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

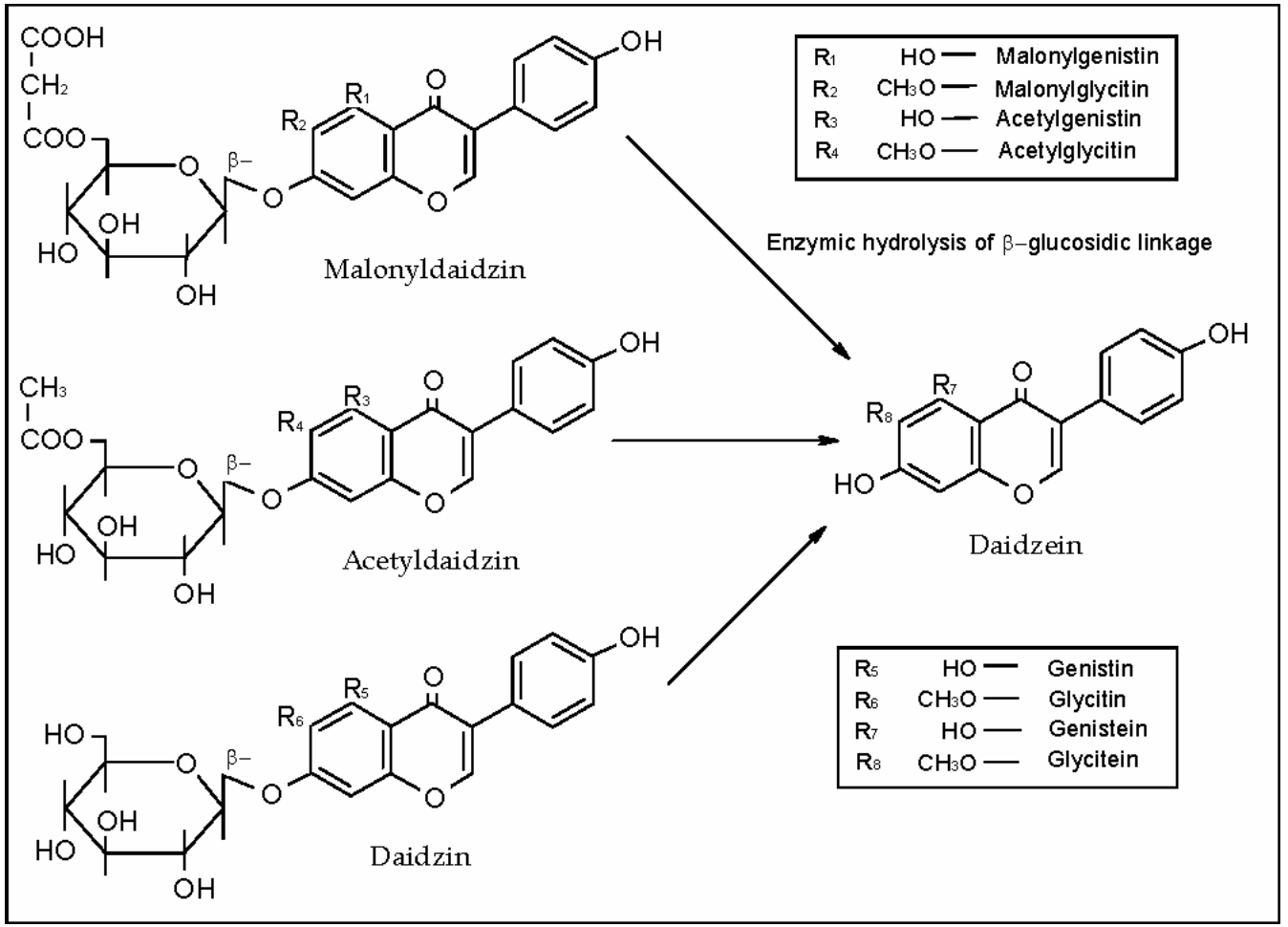

Figure 4.1 – Structural representations of isoflavone isomers and the transformations caused by bacterial-induced enzymic hydrolysis

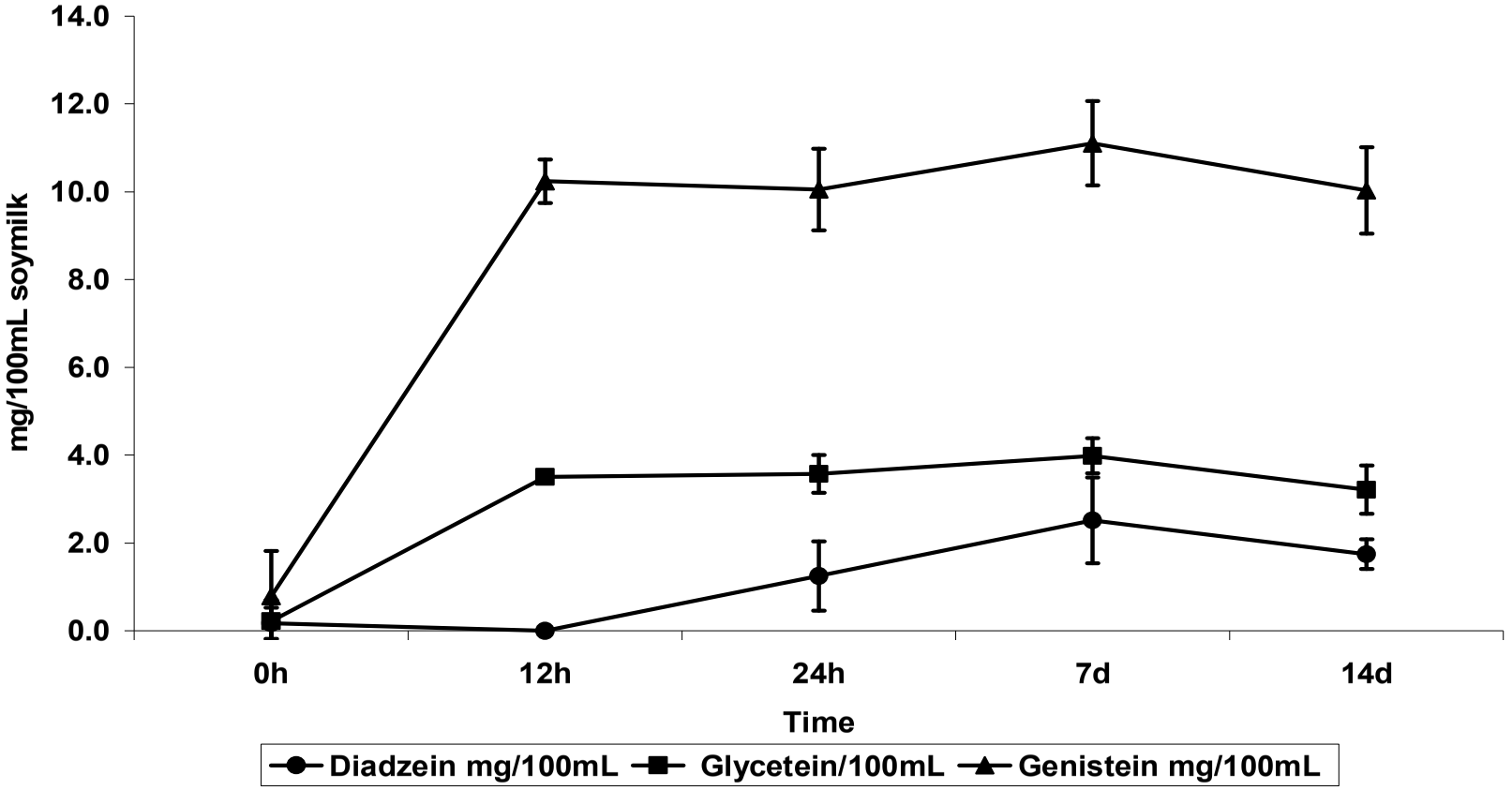

Figure 4.2 – Concentration of isoflavone aglycones in calcium fortified soymilk (mg per 100 mL) fermented with *L. acidophilus* ATCC4962 for 24 h at 37˚C then stored for 14 d at 4˚C

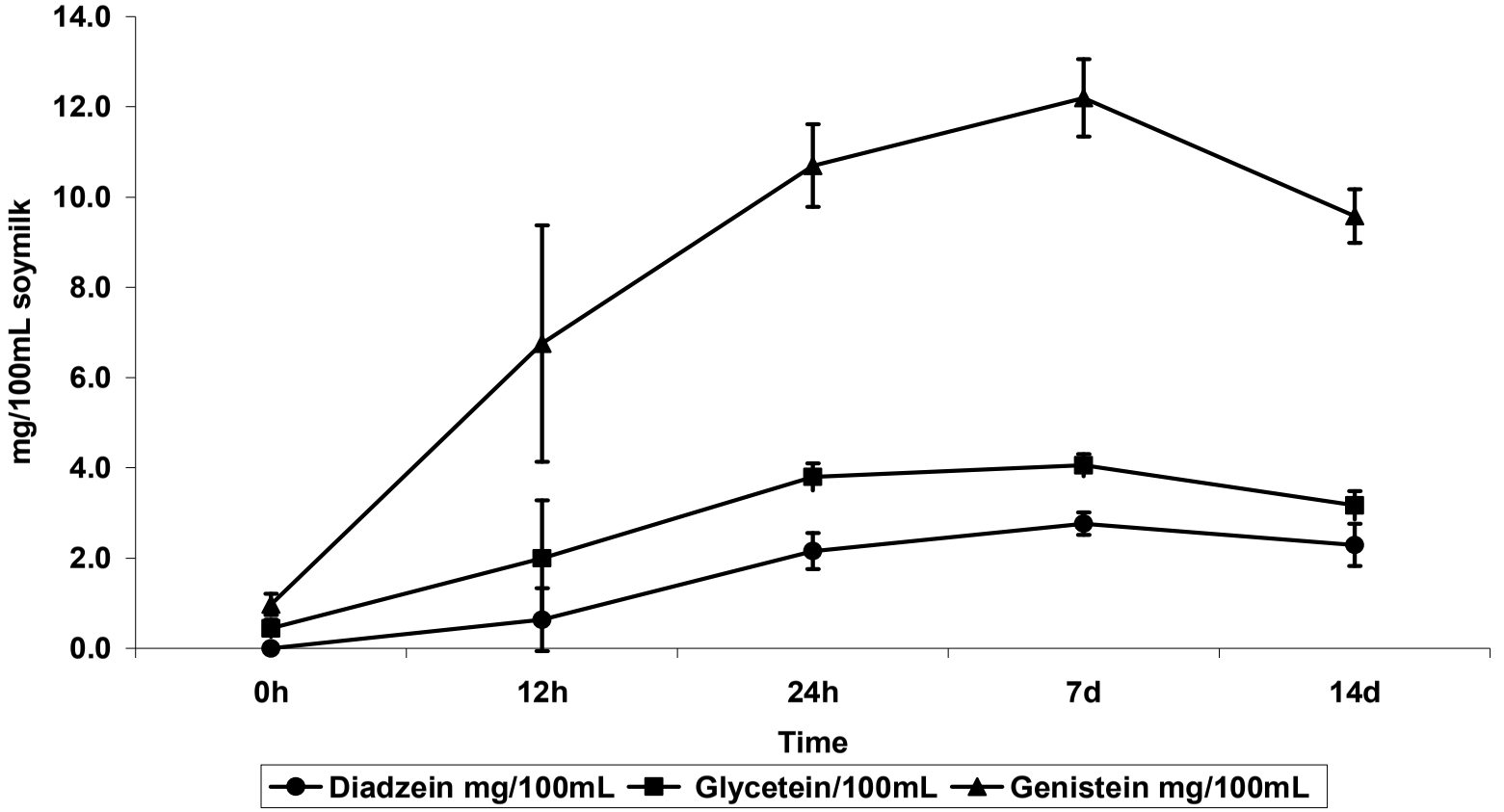

Figure 4.3 – Concentration of isoflavone aglycones in calcium fortified soymilk (mg per 100 mL) fermented with *L. acidophilus* ATCC33200 for 24 h at 37˚C then stored for 14 d at 4˚C

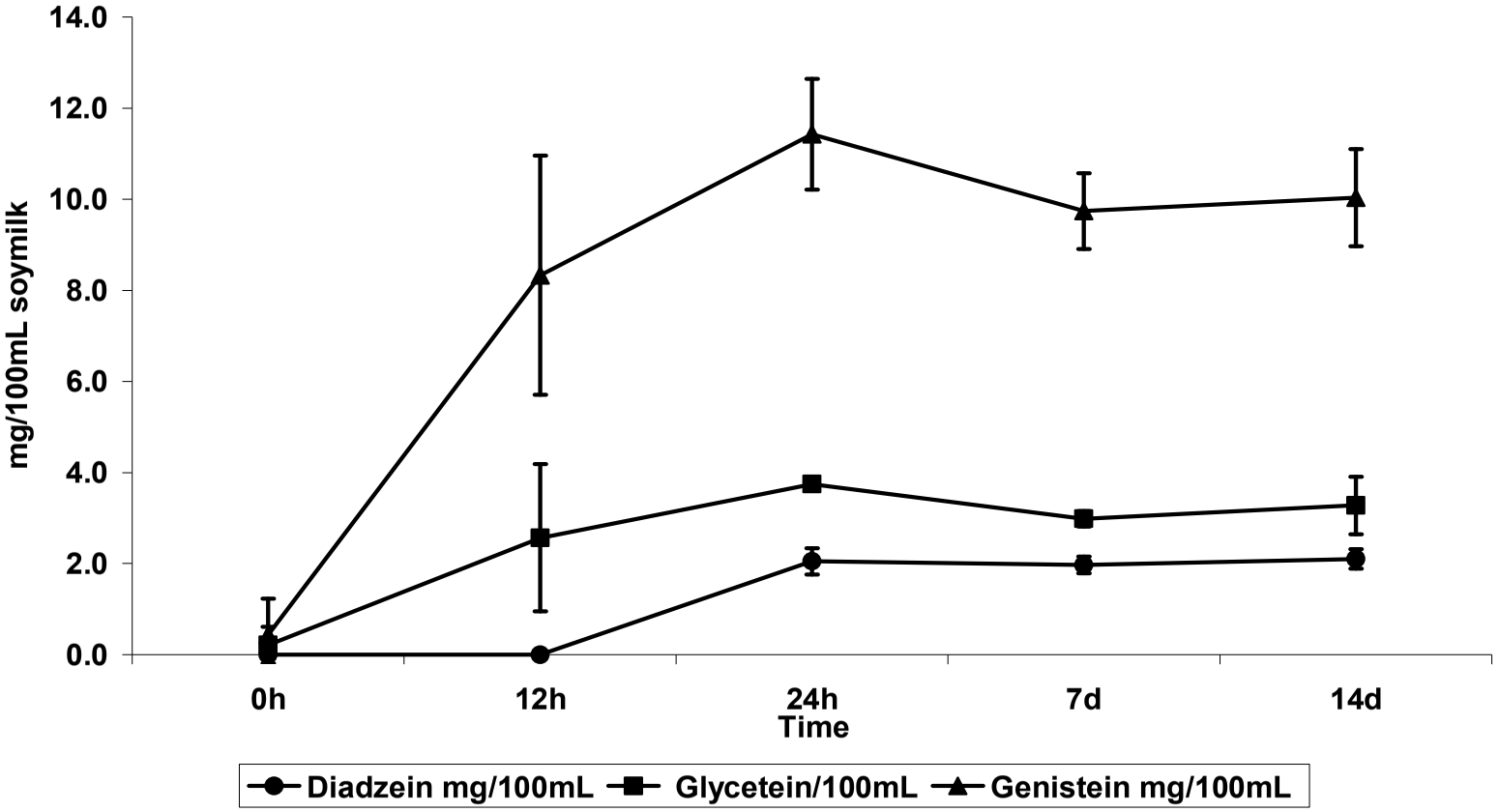

Figure 4.4 – Concentration of isoflavone aglycones in calcium fortified soymilk (mg per 100 mL) fermented with *L. casei* ASCC290 for 24 h at 37˚C then stored for 14 d at 4˚C

Chapter 5
Phytase activity from *Lactobacillus* spp. in calcium fortified soymilk and its phytic acid content[‡]

5.1 Introduction

Phytic acid (PA) is a hexaphosphoric acid ester of the 6-hydroxyl group cyclic alcohol myo-inositol. Its chemical name is myoinositol 1,2,3,4,5,6 hexa dihydrogen phosphate. The salts of phytic acid are described as phytates or inositol hexaphosphate (InsP6) (Rimbach, Pallauf, Moehring, Kraemer, & Minihane, 2008). Phytate, the salt derived from phytic acid, is a mixture of potassium, magnesium and calcium salts present as a chelate. Phytate acts as a storage form for phosphorus in cereals, legumes and oilseeds (Reddy, 2001), and is present in a wide variety of plant foods, especially in cereals and legumes including soybeans and soybean by-products such as soymilk.

Phytic acid has long been considered to have anti-nutrient properties since it can form complexes with protein and metal ions, preventing optimal mineral absorption from the intestine (Sandberg & Andlid, 2002). High phytate levels decrease the bioavailability of many minerals including iron, zinc, magnesium and calcium (Lopez, Leenhardt, Coudray, & Remesy, 2002).

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Phytase (inositol hexaphosphate hydrolase) is an enzyme which degrades phytates by dephosphorylation (Poutanen, Flander, & Katina, 2009) and hydrolysis (Noureddini & Dang, 2009). Many microorganisms exhibit phytase activity including yeasts, Bifidobacterium (Haros, Bielecka, & Sanz, 2005) and lactic acid bacteria (LAB) (Ganzle, Zhang, Monang, Lee, & Schwab, 2009). The optimal conditions for phytase activity depend on the origin of the enzyme and its available substrate.

Dephosphorylation of phytates depends on the position of the inositol ring, as well as on the formation of lower order myo-inositol phosphate ($InsP_n$) isomers such as $InsP_5$, $InsP_4$, $InsP_3$, $InsP_2$ and on features of the general biochemical environment which include: the incubation media, the pH and presence of other compounds including inhibitors and activators (Noureddini & Dang, 2009). Hydrolysis of phytic acid can help enhance mineral absorption, the fractional absorption of magnesium from white-wheat bread is significantly impaired by the addition of phytic acid, in a dose-dependent manner, at amounts similar to those naturally present in whole meal and brown bread (Bohn, Davidsson, Walczyk, & Hurell, 2004). Phytate degradation improves iron absorption from cereal porridges prepared with water but not with milk (Hurell, Reddy, Huillerat, & Cook, 2003). Another study showed that the removal of phytates from a complementary food based in wheat and soy had a beneficial effect on the fractional absorption of zinc, though not of copper (Egli, Davidsson, Zeder, Walczyk, & Hurell, 2004).

Hydrolysis of phytic acid can be achieved by the action of several different enzymes groups, including that of the phosphatases. Phosphatases catalyse the hydrolysis of phosphomonoester bonds of a wide variety of phosphate esters. Phytases constitute a subgroup of phosphotases having a general preference for phytate, which is hydrolysed to generate phosphoric acid and myo-inositol phosphates. Phytases are thus

important in human nutrition for their role in the degradation of phytate during both food processing and gastrointestinal transit (Haros, Bielecka, & Sanz, 2005). Some phytases are provided in ingested foods, and others are secreted by bacteria within the colonic microflora. Bacteria that are known to secrete phytases include: *Bacteroides* spp.*, Clostridium* spp.*, Escherichia coli, Klebsiella pneumoniae, Bifidobacterium* spp. and some *Lactobacillus* strains used in food fermentation.

Lactobacillus spp. is often used in food fermentation. These bacteria increase the shelf life and the nutritional value of many products, contributing to their unique organoleptic characteristics (Palacios, Haros, Rosell, & Sanz, 2005), and providing health benefits to consumers (Tang, Shah, Wilcox, Walker, & Stojanovska, 2007; Tsangalis, Ashton, Mcgill, & Shah, 2002). In developing functional foods and neutraceuticals, food grade lactic acid bacteria (LAB) have been studied to select types with optimal qualities for fermentation (Fumalaro, DeSimone, Pandey, Sahu, & Minisola, 2005). One study indicated that from 94 LAB strains isolated from fermented vegetable or bamboo products, 59% would degrade phytic acids. *L. plantarum* exhibited a particularly potent activity (Tamang, Tamang, Schillinger, Guigas, & Holzapfel, 2009).

Soymilk is increasingly being consumed as a milk substitute by perimenopausal women, people with lactose intolerance and vegans (Brouns, 2002; Ryan-Borchers, Soon Park, Chew, McGuire, Fournier, & Beerman, 2006b; Sadler, 2004). To ensure that soymilk is nutritionally equivalent to cows' milk, it is often fortified with calcium. The bioavailability of this added calcium may however be compromised if high levels of phytate are present.

This study aimed to investigate the phytase activity of seven strains of *Lactobacillus* spp. that are commonly used as probiotics in fermented foods. Their

phytase activity was analysed when they were incubated in culture media and also when they were fermented in a commercially available soymilk fortified with a proprietary phosphate of calcium fortificant. The amount of phytic acid was analysed. Another aim of the study was to measure the hydrolysis of the phytic acid present in calcium fortified soymilk during the fermentation process. Various methods of phytic acid detection were carried out as described in this chapter.

5.2 Materials and methods

5.2.1 Bacteria

Pure cultures of *Lactobacillus acidophilus* ATCC4962, ATCC33200, ATCC4356, ATCC4161 and *L. casei* ASCC290 were obtained from the Victoria University Culture Collection (Werribee, Victoria, Australia). In addition, *L. plantarum* ASCC276 was obtained from the Australian Starter Culture Research Centre Ltd (Werribee, Victoria, Australia) and *L. fermentum* VRI-003 from Probiomics Ltd (Sydney, NSW, Australia). The purity of cultures was checked by gram staining before organisms were stored at -80ºC in 40 % glycerol.

5.2.2 Enumeration of probiotic microorganisms in fermented milk

The pour plate method was used for bacterial enumeration using MRS agar. Plates were incubated at 37ºC for 3 days in an anaerobic jar (Becton-Dickinson Microbiology Systems ®, Sparks, MD, USA) with a gas generating kit ^{TM} (Oxoid Ltd, Hampshire, UK). Populations of probiotic microorganisms are presented as $\log_{10}$ colony forming units (CFU)/mL of calcium fortified soymilk.

5.2.3 Culture medium for measuring phytase activity

Probiotic cultures were activated through three successive transfers in modified MRS broth (De Mann, Rogosa, & Sharpe, 1960) supplemented with 0.725 g/L of phytic acid dipotassium salt $C_6H_{16}O_{24}P_6K_2$ at 37ºC for 20 h using a 5% inoculum. Bacterial growth was monitored by measuring the optical density (OD) at 600 nm and by plate count using the standard pour plate method. MRS broth was inoculated at 5% with selected bacterial strains and incubated at 37°C in anaerobic conditions. Phytase activity in the medium was measured at 12, 24, 36 and 48 h by centrifuging the cells at 4,000 × g for 15 min at 4°C and then washing with 50 mM Tris HCL (pH 6.5). The cell pellet was then suspended in 50 mM sodium acetate-acetic acid (pH 5.5) and sonicated for 20 min at 4^{o}C. An aliquot was removed and centrifuged at 14,000 × g for 15 min at 4°C, and the supernatant then underwent analysis for phytase activity and protein content.

5.2.4 Calcium fortified soymilk

The soymilk used in this study was *So Good (Sanitarium Health Food Company, NSW, Australia)*, a brand commonly consumed in Australia. It contains 4% soy protein and is fortified with a proprietary phosphate of calcium (calcium salts and phosphoric acid complexe) containing 120 mg/100 mL calcium. The phytate content is < 0.1%. To investigate phytic acid degradation, this calcium fortified soymilk was supplemented with 0.725 g/L of the phytic acid dipotassium salt $C_6H_{16}O_{24}P_6K_2$. After supplementation with phytic acid, the soymilk was fermented as described below. Aliquots of fermented soymilk were freeze dried (Dynavac FD; Airvac Engineering Pty.

Ltd., Rowville, Australia) at -20°C and -100 kPa for 72 h. The freeze dried samples were stored under vacuum at -20°C before determination of their phytic acid content.

5.2.5 Fermentation of calcium fortified soymilk

Probiotic cultures were activated through three successive transfers in MRS broth (De Man, Rogosa, & Sharpe, 1960) at 37ºC for 20 h using a 2% inoculum. Calcium fortified soymilk containing 4% soy protein and 120 mg/100 mL calcium was dispersed into 500 mL bottles and heat treated at 90ºC for 30 min. Each bottle was then inoculated with a 1% (v/v) inoculum of each probiotic microorganism before incubation at 37ºC for 24 h. Fermented soymilk samples were then stored for 14 d at 4ºC.

During the incubation period at 37°C, 100 mL aliquots of soymilk were removed aseptically from each bottle after 0 h, 12 h and 24 h. Additional aliquots were taken at 7 d and 14 d during refrigerated storage at 4°C. Viable bacterial populations were enumerated, pH was measured and phytase activity and phytate content were determined. For each organism, fermentation was performed twice and samples were measured in duplicate.

5.2.6 Phytase activity assay

Phytase activity was determined by monitoring the amount of inorganic phosphate liberated from sodium phytate (Haros, Bielecka, & Sanz, 2005). The assay mixture consisted of 400 µL of 0.1 M sodium acetate-acetic acid (pH 5.5), containing 1.2 mM sodium phytate and 200 µL enzyme extract. After incubation at 50°C for 30 min, the reaction was stopped by adding 100 µL of 20% trichloroacetic acid solution.

A sample aliquot was analysed to determine the liberated inorganic phosphate by the ammonium molybdate method where absorbance is measured at 405 nm. One unit of phytase activity (U) is defined as the amount of enzyme that produces 1 μM of inorganic phosphorus per hour at 50°C.

5.2.7 Protein quantification

The protein content in cell suspensions was determined using the Bradford method (Bradford, 1976). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when protein binding occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible colour change, within the linear range of the assay (~5-25 mg/mL)

5.2.8 Phytic acid analysis

Phytic acid was initially extracted using a method by Lehrfeld (Lehrfeld, 1994). 1 g of freeze dried samples together with 10 mL 0.5 M HCL was sonicated for 1.5 min using a sonicator (Branson Sonifier S450D, Danbury, CT, USA). 1 mL of the aliquot was diluted with 10 mL of water and placed on a silica based anion exchange column (SAX column, 75-150 μ, Alltech, Vic, Australia). The column was washed with 2 mL water and the sample was eluted with 2 mL of 2 M HCL. The eluate was evaporated to dryness in a Savant speedvac concentrator at 40°C. The residue was dissolved into 1.6 mL tetrabutylammonium hydroxide (TBNOH, 40% w/w solution in water), 0.2 mL of 5 M sulphuric acid and 0.1 mL formic acid (91%) to 100 mL of a methanol to water solution (51.5%). The solution was centrifuged at 14,000 × g for 10 min to remove any

suspended material prior to injection into the HPLC. 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 × 4.6 mm internal diameter; 5 μm) reversed-phase column was used.

The mobile phase was prepared by mixing 430 mL of acetonitrile, 570 mL of 0.035 M formic acid and 10 mL TBNOH and adjusting the pH to 4.3 with 72% sulphuric acid. The solvent was pumped through the column at a flow rate of 1.0 mL/min. When it was found that phytic acid was not detectable by this method, a modified Megazyme method (Megazyme, Ireland) was used.

In our modified megazyme assay, an acid extraction of inositol phosphate was followed by treatment with a phytase specific for phytic acid (IP6) and lower forms of myo-inositol phosphate (IP2, IP3, IP4, and IP5). Subsequent treatment with alkaline phosphatase ensures the release of the final phosphate from myoinositol phosphate (IP1) which is relatively resistant to the action of phytase. The total phosphate released is measured using a modified colourimetric method and given as grams of phosphorus per 100 g of sample material (Fiske & Subbarow, 1925; Lowry & Lopez, 1946). Phytic acid was extracted from freeze dried fermented soymilk by stirring vigorously for 4 h at room temperature with hydrochloric acid (0.66 M). The extract was then centrifuged at 14,000 × g for 10 min. An aliquot of the supernatant was neutralised by the addition of 0.5 mL of sodium hydroxide solution (0.75 M) and the neutralised sample was then used for the enzymatic dephosphorylation reaction procedure. The assay measured phosphorus released as ‘available phosphorus’ from phytic acid, myo-inositol

$(phosphate)_n$ and monophosphate esters by phytase and alkaline phosphatase. The total phosphate released was measured using a modified colorimetric method and is given as phytic acid per 100 g of freeze dried sample.

5.3 Statistical analysis

Data analysis was carried out with SPSS Inc. software (version 11.5). One-way analysis of variance (ANOVA) was used to determine significant differences between means, with the significance level taken at $\alpha = 0.05$. Tukey's HSD test was used to perform multiple comparisons between means. All data presented are mean values of two fermentations and two replicates (n = 4), unless otherwise stated.

5.4 Results and discussion

5.4.1 Enumeration of LAB in calcium fortified soymilk supplemented with 0.725 g/L phytic acid

All strains displayed viable growth in the supplemented CFSM (Table 5.1). The viability did not decrease significantly after 48 h incubation. *L. acidophilus* ATCC4962 expressed the highest viability at 24 h at 9.88 ± 0.06 $\log_{10}$ CFU/mL. These results correlate with the enumeration values obtained in Chapter 3 during fermentation at 37°C and during storage at 4°C for 14 d.

5.4.2 pH levels during phytase activity in modified MRS broth

The initial pH of the MRS broth was neutral. The pH decreased after 12 h fermentation (Table 5.2). Between 12-48 h, pH ranged from around 3.6 to 4.2. *L.*

acidophilus ATCC4962 and *L. acidophilus* ATCC4161 expressed an optimum phytase activity at 24 h when the pH reached 3.69 and 3.88, respectively.

5.4.3 Phytase activity in modified MRS broth

Figure 5.1 shows the optical density (OD) during the growth of the seven *Lactobacillus* spp. in modified MRS broth. All seven strains exhibited a similar exponential growth curve. Phytase activity was measured at selected key time points on this trajectory. Phytase activity was evident in most bacterial strains tested (Table 5.3). Peak activity occurred after 24 h incubation and decreased with time. *L. acidophilus* ATCC4962, *L. acidophilus* ATCC4356, *L. acidophilus* ATCC4161 and *L. casei* ASCC290 expressed the four highest activities, while low phytase activity was found in *L. acidophilus* ATCC33200 and *L. plantarum* ASCC276. *L. fermentum* VRI-003 showed almost no activity. Between 12 h and 24 h of fermentation, the phytase activity in cultures of *L. acidophilus* ATCC4962 and *L. acidophilus* ATCC4161 increased by 85% and 91% respectively. *L. casei* ASCC290 maintained its peak increase in phytase activity from 24 h to 48 h incubation, whereas the phytase activity of the other strains decreased after their peak at 24 h. The results confirm earlier studies indicating that some LAB strains display phytase activity (Tamang, Tamang, Schillinger, Guigas, & Holzapfel, 2009).

5.4.4 pH levels during phytase activity in calcium fortified soymilk

pH levels of the fermented calcium fortified soymilk decreased from an initial level of around 7.15 to a minimum value of 4.55 following fermentation and during the storage period of up to 14 d. All strains expressed a decrease in pH after 12 h incubation

and with another decrease occurring after 24 h. A slight decrease in pH was observed during refrigerated storage. *L. acidophilus* ATCC33200 had the highest phytase activity after 14 d storage at pH 4.92 ± 0.32, similar to that observed with *L. acidophilus* ATCC4161 at pH 4.72 ± 0.37 and *L. casei* ASCC290 at pH 4.55 ± 0.37. Those strains showed the highest phytase activity at around or slightly below pH 5 displaying stable activity in an acidic environment. Figures 5.2 and 5.3 display the relationship between pH and phytase activity during the incubation period of *L. casei* ASCC290 and *L. acidophilus* ATCC4161 respectively. The pH tended to decrease with time as the phytase activity increased. The phytase activity reached a peak after a period of time, and then the activity subsided as the pH continued to decrease.

5.4.5 Phytase activity in calcium fortified soymilk

The phytase activity of each LAB strain was then measured. Most LAB strains expressed a significant increase in phytase activity in CFSM after 24 h incubation, and this activity level was maintained even after storage of the milk for 14 d. *L. acidophilus* ATCC4161 exhibited the highest activity after 14 d storage (Table 5.4). *L. acidophilus* ATCC33200 and *L. casei* ASCC290 also displayed higher phytase activity compared to the other strains including: *L. plantarum* ASCC276 and *L. fermentum* VRI-003. These two strains displayed the lowest phytase activities. However, there was still a significant increase in phytase activity at 24 h incubation for both strains and the increase in phytase activity was maintained throughout the 14 d storage period. While *L. acidophilus* ATCC4962 had some phytase activity throughout incubation and storage time; phytase activity failed to increase significantly during that period. Figure 5.4 displays the phytase activity of five probiotic strains each fermented in CFSM showing

an increase in phytase activity at 24 h incubation. *L. acidophilus* ATCC33200 and *L. casei* ASCC290 showed a consistent increase in phytase activity until 14 d storage at 4°C.

5.4.6 Phytic acid degradation and phosphorus content in fermented calcium fortified soymilk

There was no apparent decrease in phytic acid during the fermentation of calcium fortified soymilk (Table 5.5) based on results with the modified Megazyme assay, which detected only low levels of phytic acid (IP6) throughout the fermentation period. The initial phytic acid content of around 2.8 g/100g did not decrease significantly in any of the selected strains examined. Although small decreases in phytic acid content were observed in some strains, these were not of statistical significance. Similarly, no decrease in phosphorus content was observed for any of the strains (Table 5.6).

5.4.7 Discussion

We have investigated the phytase activity of seven strains of commonly used probiotic *Lactobacillus* spp. both in modified MRS broth and in a commercially available calcium fortified soymilk fermented in our laboratory. Most strains produced phytase under both conditions, with *L. acidophilus* ATCC4161 showing the highest activity. This is consistent with a recent investigation of 40 LAB strains, where all strains tested were able to produce phytase and degrade calcium phytate (Raghavendra & Halami, 2009).

In the present study, higher phytase activities were seen when bacteria were grown in fortified soymilk than in MRS broth. *L. acidophilus* ATCC33200, for example, showed minimal activity in modified MRS broth but showed significantly higher activity in the calcium fortified soymilk. This observation may reflect the higher nutrient quality of the calcium fortified soymilk. In particular, levels of phosphorus as well as the type of carbohydrate present (lactose, glucose or fructooligosaccharides) are both known to be important for the activity of phytase in bacterial populations (Haros, Bielecka, & Sanz, 2005).

Most phytate-degrading LAB act on calcium phytate, the most abundant phytate present in cereal and legume-based foods (Raghavendra & Halami, 2009). The ability to degrade sodium phytate is much less common. In one study, only two of forty bacterial strains tested would degrade sodium phytate and then only in the presence of calcium (De Angelis, Gallo, Corbo, McSweeney, Faccia, Giovine, & Gobbetti, 2003). Some LAB strains however, including *P. pentosaceus* CFR R38 and *P. pentosaceus* CFR R35 have been reported to be able to degrade both sodium and calcium phytate (Bae, Yanke, Cheng, & Selinger, 1999; De Angelis, Gallo, Corbo, McSweeney, Faccia, Giovine, & Gobbetti, 2003; Ho, Chan, So, Yi, & Sham, 2009). One explanation why IP6 degradation was not detected in the present study when fermented with the seven strains tested may be because the phytate was added in its potassium form. Potassium phytate was used in this study to observe if potassium phytate can also be hydrolysed by phytase and can follow a similar trend to degradation observed in Ca and Na phytate.

For the bacterial strains used in the present study, the optimum pH for phytase activity was around 5. This is consistent with other studies indicating that phytase action is accelerated in an acidic environment (Poutanen, Flander, & Katina, 2009). The solubility of mineral complexes of phytic acid is related to the number of phosphates

contained per molecule. Myo-inositol phosphates ($InsP_n$) that are phosphate-rich (such as $InsP_6$ and $InsP_5$) are relatively insoluble and will decrease mineral absorption from the intestine (Haros, Carlsson, Almgren, Larsson-Alminger, Sandberg, & Andlid, 2009). As phytates are hydrolysed into lower inositol phosphates and their phosphate groupings are removed, their ability to bind minerals will decline. An exception is Ins (1, 2, 3, 6) P_4 which has been shown to enhance calcium absorption in rats (Shen, Weaver, Kempa-Steczko, Martin, Phillippy, & Heaney, 1998).

To determine the real benefits of fermenting soymilk with the LAB, it would be necessary to investigate the hydrolytic breakdown of phytate during soymilk fermentation and to identify the molecules produced. This study provides a valuable preliminary screening of LAB strains to identify some strains in which to undertake such further studies. For a long time IP6 has been known for its role in reducing mineral bioavailability. However, during phytate hydrolysis, as phosphate groups are removed from the inositol hexaphate, the mineral bonding strength decreases and solubility increases (Jackman & Black, 1951). Ins (1,2,3,6) P_4 but not Ins (1,2,5,6) P_4 fed at the same levels significantly enhanced calcium absorption from calcium ascorbate in rats (Shen, Weaver, Kempa-Steczko, Martin, Phillippy, & Heaney, 1998) indicating a possible mineral enhancing property of phytate hydrolysis during the fermentation of calcium fortified soymilk.

There are various ways of measuring phytic acid. Initially, a strong anion exchange resin method used to extract IP6 from the CFSM samples followed by HPLC analysis was chosen. Using the HPLC method, standards were eluted and a standard curve was determined. However, after sample extraction and preparation of the fermented CFSM samples, IP6 peaks could not be detected on the chromatogram. This is probably due to the low levels of phytic acid in the samples after extraction. This

method is also time consuming. After repeated trials of HPLC analysis using varied elution fluids, the Megazyme assay method was chosen as an alternative method to determine the IP6 and phosphorus level of cultured samples.

5.5 Conclusions

Fortified soymilk with LAB strains is a viable medium for phytase activity. The *Lactobacillus* spp. can produce phytase in varying degrees depending on the strain. *L. acidophilus* ATCC4161 showing the highest activity and the optimum pH for phytase activity was found to be 5. Phytase produced in both the modified MRS broth and fortified soymilk by the *Lactobacillus* spp. can potentially help hydrolyse phytate and result in the development of a specific inositol phosphate profile typical of the respective strain in the fermented soymilk. These compounds may improve the nutritional value and hence improve mineral bioavailability and bone health by the action of hydrolysed phytates action as prebiotics. Consumption of fermented soymilk with *Lactobacillus* spp. may also provide other health benefits. IP6 hydrolysis was not observed using the assay method used. Further studies are required to investigate the potential phytic acid hydrolysis by *Lactobacillus* spp. in other soymilks made from soybeans instead of soy protein isolate. Soymilk made from soybeans would have higher amounts of natural phytate.

Table 5.1 – Viable microbial count (log_{10} CFU/mL) of calcium fortified soymilk supplemented with 0.725 g/L of the phytic acid dipotassium salt $C_6H_{16}O_{24}P_6K_2$ fermented with selected probiotic microorganisms for 0 h, 12 h, 24 h, 36 h and 48 h at 37°C.

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
0 h	8.37 ± 0.04	7.84 ± 0.09	7.93 + 0.04	7.57 ± 0.38	8.40 ± 0.05	8.28 ± 0.11	8.06 ± 0.08
12 h	9.74 ± 0.13	9.48 ± 0.03	9.32 ± 0.09	9.20 ± 0.12	9.76 ± 0.08	9.56 ± 0.01	9.54 ± 0.18
24 h	9.88 ± 0.06	9.13 ± 0.02	9.40 ± 0.00	9.34 ± 0.06	9.70 ± 0.04	9.73 ± 0.05	9.82 ± 0.06
36 h	9.68 ± 0.03	9.60 ± 0.01	8.97 ± 0.10	9.08 ± 0.25	9.25 ± 0.10	9.56 ± 0.08	9.52 ± 0.05
48 h	9.23 ± 0.07	9.20 ± 0.12	9.63 ± 0.01	9.35 ± 0.01	8.30 ± 0.43	10.22 ± 0.06	9.07 ± 0.10

Results expressed as means ± standard deviation (n = 4).

Table 5.2 – pH of MRS broth supplemented with 0.725 g/L of the phytic acid dipotassium salt $C_6H_{16}O_{24}P_6K_2$ fermented with selected probiotic microorganisms for 0 h, 12 h, 24 h, 36 h and 48 h at 37˚C.

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
12 h	3.99 ± 0.04	3.98 ± 0.05	4.02 ± 0.08	4.03 ± 0.10	3.94 ± 0.05	4.10 ± 0.11	4.10 ± 0.01
24 h	3.69 ± 0.06	3.71 ± 0.03	3.91 ± 0.27	3.88 ± 0.33	3.64 ± 0.06	3.81 ± 0.09	4.10 ± 0.01
36 h	3.62 ± 0.02	3.58 ± 0.06	3.86 ± 0.36	3.86 ± 0.40	3.61 ± 0.04	3.74 ± 0.04	4.11 ± 0.02
48 h	3.61 ± 0.03	3.61 ± 0.01	3.85 ± 0.39	3.85 ± 0.42	3.59 ± 0.05	3.69 ± 0.02	4.11 ± 0.01

Results are mean ± standard deviation (n = 4).

Table 5.3 – Specific phytase activity (U per mg protein) of lactic acid bacteria cultured for up to two days in MRS broth.

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
12 h	0.11 ± 0.05^{aB}	0.10 ± 0.03^{aB}	0.10 ± 0.06^{aB}	0.05 ± 0.02^{aAB}	0.08 ± 0.05^{aAB}	0.01 ± 0.00^{aA}	0.04 ± 0.01^{aAB}
24 h	0.76 ± 0.31^{bAB}	0.03 ± 0.01^{aA}	1.16 ± 1.03^{aB}	0.58 ± 0.28^{bAB}	0.42 ± 0.26^{aAB}	0.07 ± 0.02^{cA}	0.03 ± 0.00^{aA}
36 h	0.33 ± 0.12^{abAB}	0.11 ± 0.08^{aAB}	0.32 ± 0.30^{aAB}	0.25 ± 0.10^{aAB}	0.42 ± 0.16^{aB}	0.02 ± 0.01^{abA}	0.08 ± 0.02^{bA}
48 h	0.50 ± 0.31^{abC}	0.04 ± 0.01^{aAB}	0.39 ± 0.08^{aBC}	0.20 ± 0.08^{aABC}	0.43 ± 0.21^{aC}	0.03 ± 0.01^{bA}	0.04 ± 0.01^{aAB}

Results are given as the mean ± standard deviation (n = 4). Means in column with different lowercase letters are significantly different ($P < 0.05$). Means in rows with different upper case superscripts are significantly different ($P < 0.05$).

Table 5.4 – Specific phytase activity (U per mg protein) of calcium fortified soymilk fermented with selected probiotic microorganisms for 12 h and 24 h at 37 °C and then stored for 7 d and 14 d at 4 °C

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
0 h	0.46 ± 0.03^{aABC}	0.51 ± 0.01^{aC}	0.42 ± 0.00^{aABC}	0.44 ± 0.06^{aABC}	0.40 ± 0.02^{aAB}	0.46 ± 0.00^{aABC}	0.37 ± 0.08^{aA}
12 h	0.55 ± 0.07^{aB}	0.53 ± 0.13^{aAB}	0.61 ± 0.18^{abB}	0.48 ± 0.06^{aAB}	0.52 ± 0.06^{aAB}	0.48 ± 0.12^{abAB}	0.30 ± 0.02^{aA}
24 h	0.82 ± 0.22^{aA}	0.96 ± 0.18^{abA}	1.00 ± 0.37^{bA}	1.01 ± 0.32^{abA}	0.93 ± 0.30^{abA}	0.69 ± 0.04^{cA}	0.66 ± 0.12^{bA}
7 d	1.12 ± 0.26^{aAB}	1.35 ± 0.37^{bB}	0.87 ± 0.19^{abAB}	1.00 ± 0.17^{abAB}	1.15 ± 0.38^{abAB}	0.72 ± 0.01^{cA}	0.65 ± 0.05^{bA}
14 d	0.97 ± 0.65^{aA}	1.57 ± 0.53^{bA}	0.60 ± 0.14^{abA}	1.65 ± 0.99^{bA}	1.38 ± 0.65^{bA}	0.65 ± 0.13^{bcA}	0.71 ± 0.18^{bA}

Results are mean ± standard deviation (n = 4). Means in column with different lowercase letters are significantly different ($P < 0.05$). Means in rows with different upper case superscripts are significantly different ($P < 0.05$).

Table 5.5 – Phytic acid (g/100g) content of freeze dried calcium fortified soymilk supplemented with 0.725 g/L of the phytic acid dipotassium salt $C_6H_{16}O_{24}P_6K_2$ fermented with selected probiotic microorganisms for 0 h, 12 h, 24 h, 36 h and 48 h at 37°C.

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
0 h	2.23 ± 0.41 ^{aA}	2.45 ± 0.88 ^{aA}	3.61 ± 2.44 ^{aA}	2.28 ± 0.55 ^{aA}	3.07 ± 0.34 ^{aA}	3.15 ± 1.01 ^{aA}	2.29 ± 0.94 ^{aA}
12 h	2.96 ± 0.81 ^{aA}	3.02 ± 0.90 ^{aA}	2.63 ± 1.43 ^{aA}	1.93 ± 0.43 ^{aA}	3.34 ± 0.40 ^{aA}	3.41 ± 0.28 ^{aA}	2.24 ± 0.13 ^{aA}
24 h	2.9 ± 0.47 ^{aA}	3.2 ± 1.25 ^{aA}	2.82 ± 1.36 ^{aA}	2.36 ± 2.32 ^{aA}	3.57 ± 0.29 ^{aA}	3.31 ± 0.24 ^{aA}	1.30 ± 1.54 ^{aA}
7 d	5.3 ± 2.29 ^{aA}	3.22 ± 0.55 ^{aA}	3.55 ± 0.41 ^{aA}	3.77 ± 0.09 ^{aA}	3.12 ± 0.10 ^{aA}	3.36 ± 0.84 ^{aA}	3.91 ± 1.20 ^{aA}
14 d	2.61 ± 1.13 ^{aA}	3.82 ± 0.58 ^{aA}	4.77 ± 1.27 ^{aA}	3.53 ± 0.21 ^{aA}	2.56 ± 1.09 ^{aA}	3.00 ± 0.61 ^{aA}	3.19 ± 1.00 ^{aA}

Results are given as the mean ± standard deviation (n = 2). Means in column with different lowercase letters are significantly different ($P < 0.05$). Means in rows with different upper case superscripts are significantly different ($P < 0.05$).

Table 5.6 – Phosphorus content (g/100g) of freeze dried calcium fortified soymilk supplemented with 0.725 g/L of the phytic acid dipotassium salt $C_6H_{16}O_{24}P_6K_2$ fermented with selected probiotic microorganisms for 0 h, 12 h, 24 h, 36 h and 48 h at 37°C.

Time	*L. acidophilus* ATCC4962	*L. acidophilus* ATCC33200	*L. acidophilus* ATCC4356	*L. acidophilus* ATCC4161	*L. casei* ASCC290	*L. plantarum* ASCC276	*L. fermentum* VRI-003
0 h	0.63 ± 0.12 ^{aA}	0.69 ± 0.24 ^{aA}	1.01 ± 0.70 ^{aA}	0.64 ± 0.16 ^{aA}	0.86 ± 0.10 ^{aA}	0.89 ± 0.28 ^{aA}	0.64 ± 0.03 ^{aA}
12 h	0.84 ± 0.23 ^{aA}	0.85 ± 0.25 ^{aA}	0.74 ± 0.41 ^{aA}	0.54 ± 0.12 ^{aA}	0.94 ± 0.11 ^{aA}	0.96 ± 0.08 ^{aA}	0.63 ± 0.04 ^{aA}
24 h	0.84 ± 0.13 ^{aA}	0.90 ± 0.35 ^{aA}	0.79 ± 0.38 ^{aA}	0.66 ± 0.65 ^{aA}	1.01 ± 0.08 ^{aA}	0.93 ± 0.07 ^{aA}	0.37 ± 0.52 ^{aA}
7 d	1.49 ± 0.65 ^{aA}	0.91 ± 0.15 ^{aA}	1.00 ± 0.11 ^{aA}	1.06 ± 0.03 ^{aA}	0.88 ± 0.03 ^{aA}	0.95 ± 0.24 ^{aA}	1.10 ± 0.34 ^{aA}
14 d	0.73 ± 0.32 ^{aA}	1.07 ± 0.16 ^{aA}	1.34 ± 0.36 ^{aA}	1.00 ± 0.06 ^{aA}	0.72 ± 0.31 ^{aA}	0.85 ± 0.17 ^{aA}	0.89 ± 0.85 ^{aA}

Results are given as the mean ± standard deviation (n = 2). Means in column with different lowercase letters are significantly different ($P < 0.05$). Means in rows with different upper case superscripts are significantly different ($P < 0.05$).

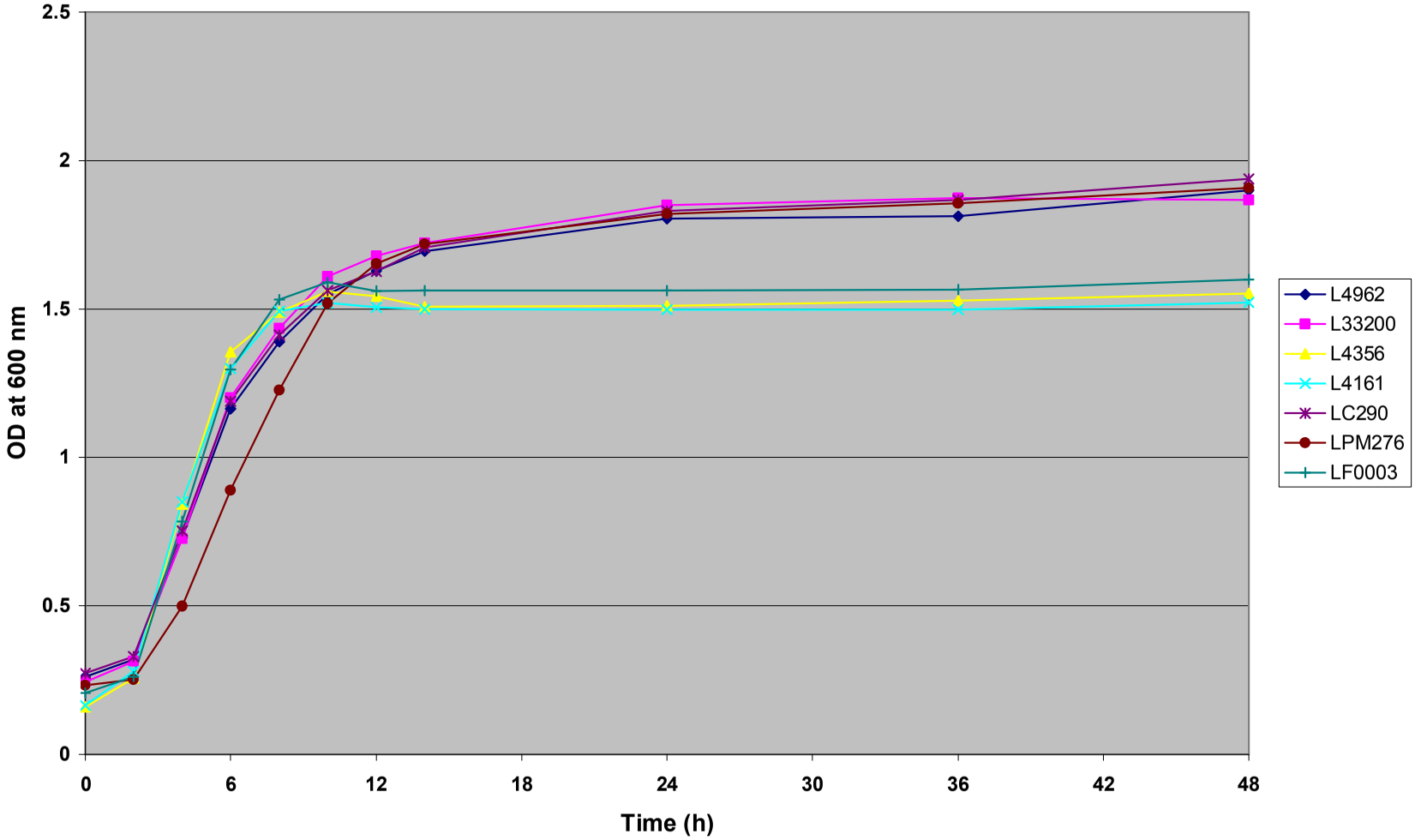

Figure 5.1 – Optical Density (OD) at 600 nm during growth of *Lactobacillus* spp. in modified MRS broth

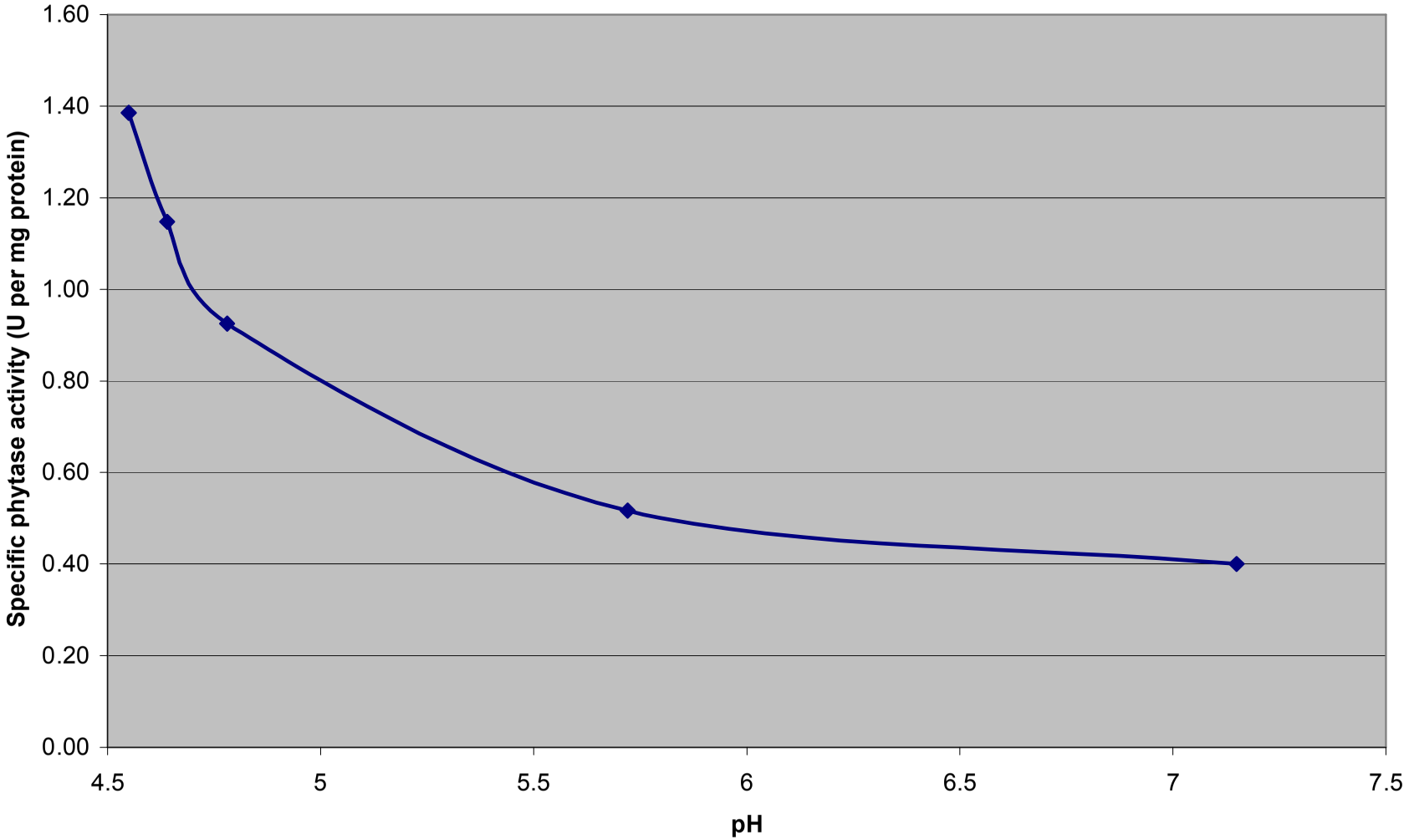

Figure 5.2 – Relationship between pH and phytase activity of *L. casei* ASCC290 in CFSM

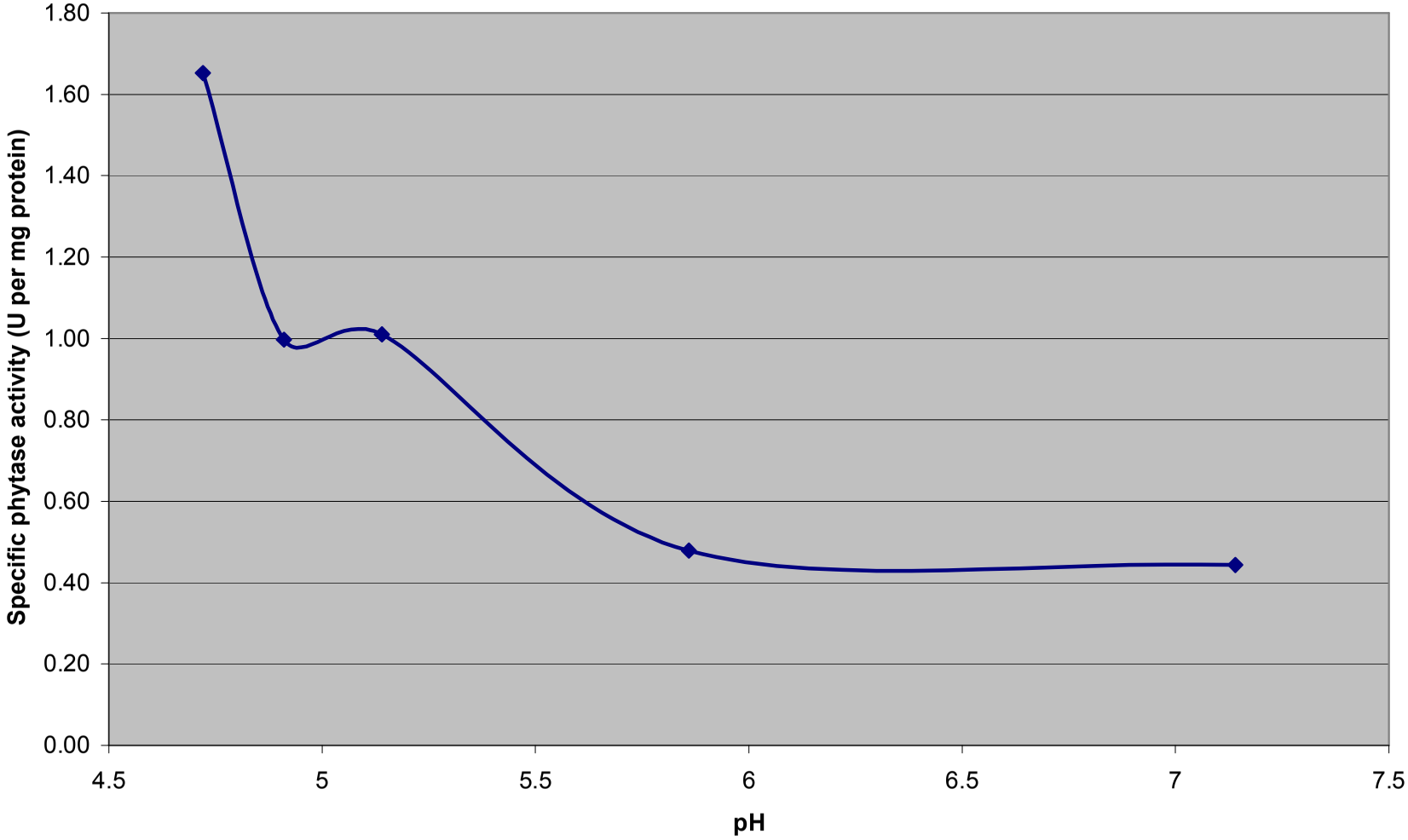

Figure 5.3 – Relationship between pH and phytase activity of *L. acidophilus* ATCC4161 in CFSM

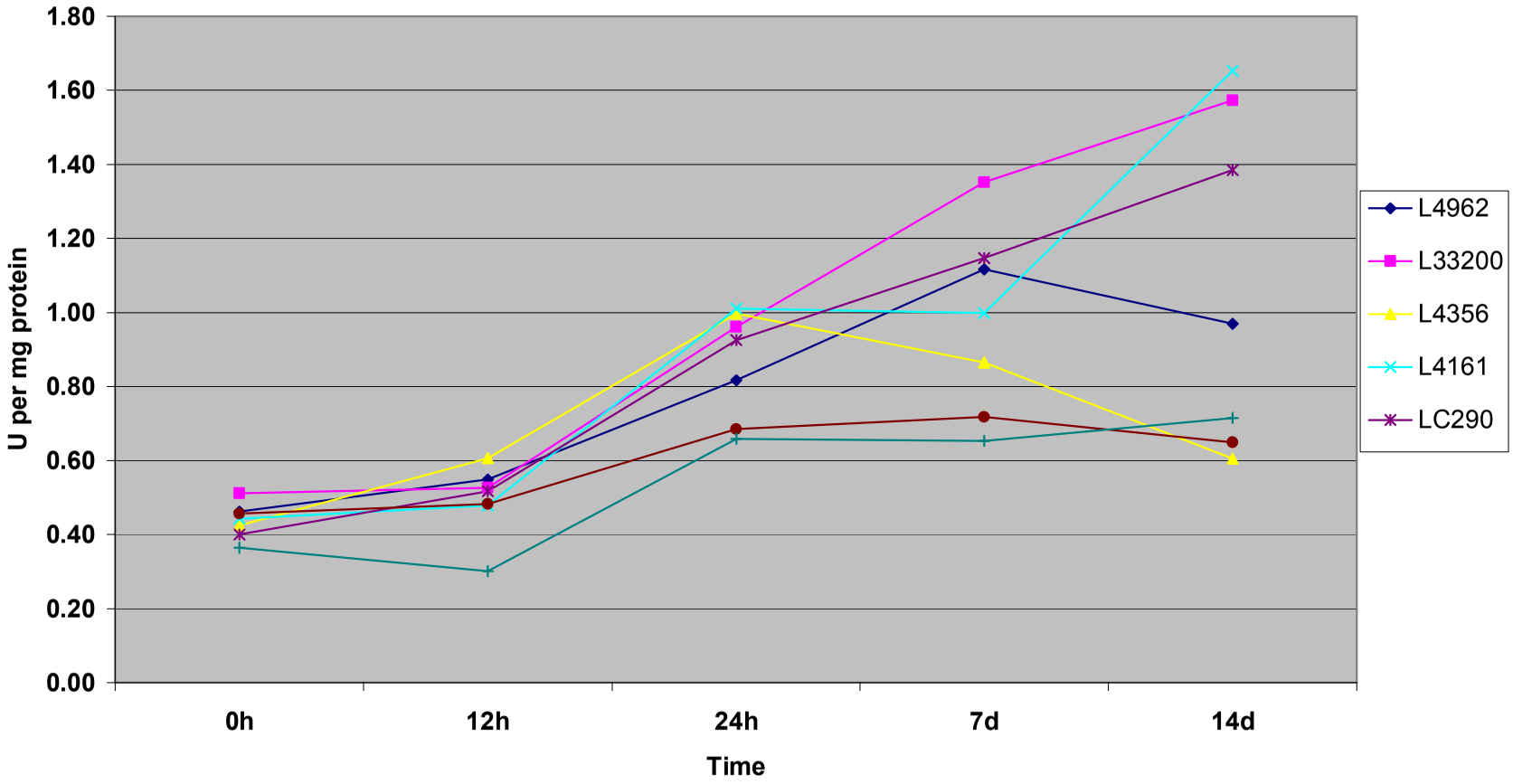

Figure 5.4 – Specific phytase activity of probiotic strains in fermented soymilk (U per mg protein)

Chapter 6
Labelling of ^{45}Ca radioisotope in calcium fortified soymilk and the electrophoresis of ^{45}Ca labelled soy protein[§]

6.1 Introduction

Soymilk is often fortified with calcium, although methods of fortification vary considerably between products (Heaney, Rafferty, & Bierman, 2005). The method of fortification has importance as calcium bioavailability will depend considerably on the choice of fortificant (Zhao, Martin, & Weaver, 2005). To date, few studies have compared absorption of calcium from the different kinds of fortified soymilk available on the market (Heaney, Dowell, Rafferty, & Bierman, 2000; Zhao, Martin, & Weaver, 2005).

In order to measure absorption of a micronutrient such as calcium from the diet requires the use of radioactive or stable isotopes to label the trace-element source (Patterson & Veillon, 2001). Isotopic labelling of calcium in a test food will then allow differentiation between the proportion of calcium present in the test food from that derived from other sources (either dietary or endogenous in origin) and present in body

[§] Part of this chapter has been published. Tang, A. L., Walker, K. Z., Wilcox, G., Strauss, B. J., Ashton, J. F., & Stojanovska, L. (2010).Calcium absorption in Australian osteopenic post menopausal women: An acute comparative study of fortified soymilk to cows' milk. *Asia Pacific Journal of Clinical Nutrition*, 19(2), 243-249.

fluids and tissues (Fairweather - Tait, Fox, Harvey, Teucher, & Dainty, 2001). Any isotope used to label a test food should be present in the same chemical form as the native trace element. While this can be achieved by biosynthetic (intrinsic) labelling, this method is generally expensive, impractical and time consuming (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998).

An alternative strategy is extrinsic labelling where the isotope is simply mixed with the food until complete isotopic exchange occurs. The radioisotope most commonly used to measure calcium absorption is ^{45}Ca (Nordin, Morris, Horowitz, Coates, O'Loughlin, & Need, 2009; Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998; Nordin, Need, Morris, O'Loughlin, & Horowitz, 2004). A study by Heaney and coworkers, however, has found that extrinsic labelling of soymilk with ^{45}Ca did not produce a uniform tracer distribution throughout the liquid and solid phases. This poor distribution resulted in a 50% overestimate of true absorbability, leading to the conclusion that an intrinsic method of labelling should be used to study the bioavailability of calcium fortificants in such liquid suspensions (Heaney, Dowell, Rafferty, & Bierman, 2000).

The great majority of calcium present in calcium fortified soymilk (CFSM) originates from the fortificant itself and not from the milk. Calcium bioavailability studies on CFSM therefore require that the fortificant is well labelled with ^{45}Ca. In this present study, the process of intrinsic labelling of the fortificant has been termed: ‘labelling of the fortificant prior to soymilk manufacture’, while extrinsic labelling, the least time consuming and simpler method, has been termed: ‘labelling of the fortificant after soymilk manufacture’. This study compares the calcium distribution in the CFSM system between these two methods of labelling, to determine if extrinsic labelling can

be used, being the most simple and efficient way of tracer labelling for calcium absorption studies on soymilk.

One of the factors that affect calcium absorption and bone mass is the amount of dietary protein consumed. Increased protein consumption may increase the excretion of urinary calcium (Kerstetter, O'Brien, Caseria, Wall, & Insogna, 2005), although this may be offset by increases in absorption. Protein is a major component of bone matrix and there are debates in the literature over the effect of dietary protein on bone mass (Vatanparast, Bailey, Baxter-Jones, & Whiting, 2007). Both calcium and protein intake must be adequate to fully realise the benefit of each nutrient on bone (Heaney & Layman, 2008). In soymilk, four major soy protein fractions have been identified, each classified according to their sedimentation properties. The 2S, 7S, 11S and 15S fractions comprise 8%, 35%, 52% and 5% of the total protein content respectively (Khatib, Herald, Aramouni, MacRitchie, & Schapaugh, 2002). The 7S globulin fraction (β-conglycinin) and the 11S fraction (glycinin) are storage proteins accounting for 65-80% of total seed proteins used to manufacture the soy protein isolate used in soymilk. β-conglycinin has three main subunits designated as α', α and β with molecular weights (MW) of 57, 57 and 42 kDa, respectively (Liu, 1997). Glycinin (11S) has a MW of about 350 kDa. It is composed of acidic polypeptide chains with MW of 37-42 kDa and basic (B) polypeptide chains with MW of 20 kDa, paired by disulfide bonds (Khatib, Herald, Aramouni, MacRitchie, & Schapaugh, 2002). Soymilk coagulates when calcium is added and the pH is decreased (Ono, Katho, & Mothizuki, 1993). This study therefore aims to compare two methods by which to label CFSM with ^{45}Ca, and to examine how the isotope becomes bound to soymilk proteins using electrophoresis.

6.2 Experimental methods

6.2.1 Calcium fortified soymilk

The CFSM used in this study is widely sold throughout Australia (*So Good, Sanitarium Health Foods, NSW, Australia*). It is made from soy protein (4%) and has been fortified to achieve similar calcium content to cows' milk (120 mg/100 mL).

6.2.2 Labelling of fortificant with ^{45}Ca prior to soymilk manufacture

The main source of calcium in CFSM comes from the added fortificant. Labelled salts of the proprietary phosphate of calcium fortificant used in *So Good* were prepared by dissolving the amount of the proprietary salt required to yield 120 mg of calcium/100 mL in water before adding one microgram of $^{45}CaCl_2$ (Amersham Biosciences, England). This solution was kept at 90°C for 24 h to evaporate to dryness. The dry salts were then ground into a fine powder using a mortar and pestle, and added to 100 mL of unfortified soymilk. Fortified soymilk samples were blended at high speed for 5 min, heat treated for 30 min at 90°C and stored at 4°C for 24 h. Each sample had a final total tracer concentration of ≈ 185 kBq/100 mL.

6.2.3 Labelling of fortificant with ^{45}Ca after soymilk manufacture

The fortificant present in CFSM was labelled by adding one microgram of high-specific-activity $^{45}CaCl_2$ to a known amount of soymilk sample, yielding a tracer concentration of ≈ 185kBq/100 mL. Labelled milk was vortexed continuously for 1 min and then stored immediately at 4°C or heat treated (90°C for 30 min) before storing at 4°C for 24 h to allow for calcium exchange to occur.

6.2.4 Fractionation of labelled soymilk samples

CFSM that had been labelled (as above) either before or after soymilk manufacture were fractionated to determine the ^{45}Ca distribution as described by Heaney and coworkers (Heaney, Dowell, Rafferty, & Bierman, 2000). Briefly, CFSM aliquots in 1.5 mL tubes were centrifuged for 30 min at 14,000 × g (Eppendorf centrifuge, model 5415C, Crown Scientific Pty. Ltd., Vic, Australia). Each sample was then separated into four portions: the upper fat layer, an intermediate soluble fluid layer, the remaining supernatant and the pelleted residue. The upper fat layer was discarded and the supernatant portion was analysed for both ^{40}Ca and ^{45}Ca, as detailed below. Samples of unfractionated CFSM were also analysed in a similar way to allow calculation of the ^{40}Ca and ^{45}Ca content of the residual insoluble portion (Heaney, 2001).

6.2.5 Calcium analysis

Fractionated and unfractionated samples (0.1g unfractionated and 0.5g fractionated samples for ^{40}Ca content measurement and 1g both fractionated and unfractionated samples for ^{45}Ca measurement) were heated at 550°C in a muffle furnace and the resulting ashes were dissolved in hydrochloric acid (0.5 mol/L). ^{40}Ca content was determined by diluting the ash sample with 5 mL of 0.5 mol/L HCL containing 0.5% lanthanum as $LaCL_3$ before analysis by atomic absorption spectrophotometry (Varian SpectrAA-300/400, Palo Alto, Ca, USA). ^{45}Ca was measured from ash samples distributed in Ecoscint scintillation fluid (National Diagnostics, UK) using a liquid scintillation counter (Wallac 1410, Perkin Elmer Life Sciences, Massachusetts, USA).

6.2.6 Electrophoresis of calcium fortified soymilk labelled with ^{45}Ca

The fortificant was labelled with ^{45}Ca after CFSM manufacture to give a concentration of 185 kBq/mL, as stated in Section 6.2.3. Labelling was followed by a heat treatment of 90°C for 30 min. The sample was then kept at 4°C for 24 h to allow for calcium exchange.

The calcium binding property of soy proteins was analysed by SDS-PAGE using the stacking gel system by Laemmli (Laemmli, 1970), as described by Ong *et al*. (Ong, Henriksson, & Shah, 2006). An aliquot of labelled CFSM (300 μL) was suspended in a mixture of 1 mL Tris (10mM) (Sigma-Aldrich) – EDTA (1 mM) pH 8.0 buffer, 400 μL of 10% SDS (Sigma-Aldrich) and 50 μL of β-mercaptoethanol (Bio-Rad Laboratories Ltd., Watford, UK). The samples were boiled for 5 min twice at 5 min intervals. A 25 μL aliquot of stock solution was diluted with 100 μL of treatment buffer (0.125 M Tris-chloride, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue (pH 6.8) (Sigma-Aldrich). Whole casein was dissolved in water at pH 10 and α-, β-, κ-caseins (Sigma-Aldrich) were dissolved in water at neutral pH to a concentration of 2 mg/mL. A 20 μL aliquot of this mixture was diluted with 40 μL of treatment buffer.

A working volume (10 μL) of each sample and 7.5 μL of each control (the α-, β-, κ- and whole caseins) were loaded into pre-cast 12% polyacrylamide electrophoresis gels (LifeGels, Life Therapeutics, Frenchs Forest, NSW, Australia). A 10 μL volume of broad range, pre-stained SDS-PAGE standards (Bio-Rad) was used as a marker. Gels were run in a BIO-RAD Mini-Protean® II slab cell filled with tank buffer solution (0.025 M Tris, 0.192 M glycine, 0.1% SDS (pH 8.3), run at 50 mA by a Power Pac 300. Gels were fixed in de-staining solution I (40% methanol, 7% acetic acid) for 30 min before staining with staining solution (0.025% Coomassie Brilliant Blue, (ICN

Biochemicals Inc., Aurora, OH, USA), 40% methanol, 7% acetic acid) for 4 h. The gels were then de-stained in de-staining solution I for 1 h followed by de-staining in de-staining solution II (7% acetic acid, 5% methanol) until the background became clear. Gel images were recorded using an Intelligent Dark Box II (Fuji Photo Film Co. Ltd., Tokyo, Japan) with LAS-1000 Lite V1.3 software (Fuji Photo Film Co. Ltd, Tokyo, Japan). The area and intensity of the bands were measured and detected using Image Gauge V4.0 software (Fuji Photo Film Co. Ltd, Tokyo, Japan).

6.2.7 Detection of ^{45}Ca on the gels

A Fluorescent Image Analyser FLA-3000 (Fuji) was used to detect the presence of radioactive ^{45}Ca when gels were exposed to an Imaging Plate (IP). In brief, the FLA-3000 uses a solid-state laser emitting a wavelength that can excite a fluorochrome. By employing multiple lasers of different wavelengths combined with four types of filters, it is possible to read fluorochrome-stained gels, while the Imaging Plate (IP) can also act as a radioactive energy sensor. After recording the image of the gels, they were dried for 2 h, then placed into the IP for 6 h to allow for sufficient radioactive exposure. The IP was then placed to the FLA-3000 for radioactivity detection.

Other gels were prepared as above, but were not dried. In this case the predominant soy protein bands were cut out in order to determine the amount of radioactive ^{45}Ca present. Each soy protein band was dissolved by the addition of 1 mL of either 6% or 30% hydrogen peroxide (H_2O_2), then incubated at 60°C overnight. 10 mL of Starscint scintillation fluid (Perkin Elmer) was then added to the vial and the radioactivity present was measured using a liquid scintillation counter (Wallac 1410, Perkin Elmer Life Sciences, Massachusetts, USA). A similar sized part of the gel

without a protein band present was taken as blank. Data from the analysis were counts per minute per mg (cpm/mg) of gel sample.

6.3 Statistical analysis

Fortificant labelling before soymilk manufacture was performed once in triplicate, while labelling of fortificant after soymilk manufacture (with and without heat treatment) was performed on two occasions, also in triplicate. Results are presented as mean ± standard deviation. Differences in the amount of tracer calcium relative to stable calcium were compared using univariate analysis of variance (ANOVA). Multivariate analysis of variance was used for multiple comparisons. SPSS for Windows (Version 11.5, SPSS Australasia Ltd, Melbourne) was used for all statistical analyses. A P value of < 0.05 was taken as significant.

6.4 Results and discussion

6.4.1 Source labelling

Atomic spectroscopy established that only 21-22 % of the stable calcium (^{40}Ca) present in samples of CFSM could be detected in the supernatant fraction, even following 24 h storage at 4°C (Table 6.1). When the fortificant was labelled with ^{45}Ca before it was added to the soymilk, the relative abundance of ^{45}Ca in the supernatant was found to be only slightly (4-5 %) but not significantly ($P > 0.05$) lower than that of the stable isotope at each time point measured. In contrast, when the fortificant was labelled after it had been added to the soymilk, the relative abundance of calcium tracer in the supernatant was significantly ($P < 0.05$) higher than that of stable calcium (^{40}Ca),

even following 24 h storage. When additional heat treatment was applied however, the abundance of ^{45}Ca in the supernatant was now reduced to a level very similar to that of stable calcium. Tracer abundance continued to remain at this level after 24 h at 4°C.

The percentage of radioactive tracer in the supernatant and in the residual portion of CFSM samples using the three different labelling methods is given in Figure 6.1. This figure indicates that provided a heat treatment was applied, the labelling of fortificant after its addition to soymilk resulted in tracer abundance very similar ($P < 0.05$) to that found when fortificant labelling was carried out prior to the soymilk manufacture. Given the relative ease of labelling CFSM after manufacture, this method with heat treatment was used for our *in vivo* study detailed in Chapters 7 and 9 following.

6.4.2 Electrophoresis of labelled CFSM after soymilk manufacture

The three main β-conglycinin (7S) globulins found in the soy beans were identified during the electrophoresis of labelled CFSM: α'β-conglycinin, αβ-conglycinin and β-glycinin with MW ~ 81, 57 and 42 kDa. The hexametric molecule glycynin was also identified as the acidic (40 kDa) and basic (20 kDa) polypeptide chains. A broad range prestained MW standard was used as reference (Figure 6.2).

6.4.3 Radioactivity (cpm/mg Gel) of SDS PAGE soy protein labelled with ^{45}Ca

The presence of radioactive ^{45}Ca in the electrophoretic gels could not be detected on the Imaging Plate (IP) of the FLA-3000 analyser (Fuji) after a 6 h exposure. Detection could not be improved by increasing the exposure time to 12 h or by

increasing the dose of radioactivity ten-fold. For this reason, the alternative method of counting radioactivity in excised gel bands was undertaken.

Table 6.2 shows that there are no significant differences in cpm/mg between the different banded sections of the gel. Therefore, it appears that ^{45}Ca is evenly distributed throughout the gel, and the ^{45}Ca does not bind with any of the three major proteins: α' β-conglycynin, α-β-conglycinin and β-glycinin in the labelled CFSM. Dissolving the gel in 30% H_2O_2 increased the cpm/mg count to about double the cpm/mg count when the gel is dissolved in 6% H_2O_2. The figures presented in Table 6.2 are not above background radiation; therefore, no conclusion can be drawn from these data.

6.4.4 Discussion

Bioavailability studies require appropriate distribution of radioisotope label in the test food. Tracer methods to measure calcium absorption are not commonly used for marketed products because they often cannot be easily labelled (Heaney, 2003). This study has shown that provided that a heat treatment is applied, a calcium fortificant can be labelled for tracer studies after it has been added to soymilk. This method will then generate tracer abundance very similar to that obtained when the fortificant has been labelled prior to its addition to soymilk. This is a valuable observation pointing to a practical method that can be usefully applied to compare calcium absorption across the range of commercially available CFSMs when access to the initial fortificant is not necessarily available. It would be very valuable in establishing the calcium bioavailability from different types of fortified soymilks or fortified drinks since it has been shown that not all calcium fortified beverages including soymilk are equivalent (Heaney, Rafferty, & Bierman, 2005).

Earlier studies on cows' milk and other dairy products (Nickel, Berdine, Smith, Smith, Miller, & Weaver, 1996) as well studies on a calcium-containing wheat flour product (Weaver, Heaney, Martin, & Fitzsimmons, 1992) have indicated that for these foods, labelling of fortificant after its addition to the food can generate a similar tracer abundance to that obtained when the fortificant is labelled before it is added to the food. For foods such as cows' milk, a calcium isotope appears to be able to exchange readily with stable calcium unheeded by the physical and chemical nature of the food. In contrast, when heat treatment is not used, fortificant labelling after addition to foods such as green leafy vegetables (Heaney, Weaver, & Recker, 1988) or soymilk (Heaney, Dowell, Rafferty, & Bierman, 2000) gives rise to non-uniform tracer distribution, and for CFSM a 50% overestimation of true absorbability (Heaney, Dowell, Rafferty, & Bierman, 2000). Our study indicates that this problem can be avoided by heat treatment. Heat treatment may promote an exchange between soluble calcium and insoluble calcium. Insoluble calcium is the predominant form of calcium in both the proprietary fortificant added to *So Good*, and in CFSM labelled after manufacture (> 80%). This heat treatment method will therefore be used in our *in vivo* bioavailability studies.

The soy proteins identified from the CFSM sample were a mixture of β-conglycinin and β-portion of glycinin, and they are the main soy proteins from soybeans. However, using this method the smaller MW soy proteins (< 20 kDa) could not be identified. Casein phosphopeptides of cows' milk have been found to dissolve calcium and thus promote its absorption in growing rats by combining phosphoserines (from phosphopeptides) with calcium to form amorphous tricalcium phosphate nanoclusters (Bao, Song, Zhang, Chen, & Guo, 2007). Soy protein hydrolysates (SPH) are able to promote calcium absorption and a diet rich in soy protein has been shown to increase intestinal calcium absorption in women and it was demonstrated that some soy

protein hydrolysates can bind calcium using different proteases. The peptide fragments that exhibited high calcium binding capacity had molecular weights of either 14.4 or 8-9 kDa and the level of Ca-bound increased linearly with the increment of carboxyl content in the SPHs and further deamidation of the SPHs from protease improved Ca-binding of the hydrolysate (Bao, Song, Zhang, Chen, & Guo, 2007).

Soymilk usually coagulates upon the addition of calcium and decreasing pH, and this coagulation results in the binding of calcium to soy protein and phytate (Ono, Katho, & Mothizuki, 1993). This study could not demonstrate any ^{45}Ca binding to the soy proteins using the two methods outlined previously. The possible causes are that the soy protein separated during electrophoresis could not bind with the ^{45}Ca since they were of broad range (80 – 20 kDa) and the SPHs known to bind with calcium were not identified and tested. The peptides fragments known to bind with calcium were not present in the CFSM due to the source and nature of the soy protein isolate used to manufacture the product and the pH of the product is neutral (~ pH 7). Therefore, the calcium does not bind with the SPHs present in the CFSM system and it is possibly the reason why most of the fortificant is present in the insoluble part.

The radioisotope gel labelling methods used to identify possible calcium binding properties of the soy protein present in the CFSM had never been trialled before. It is important to note that more time and trials are needed on this new idea to identify the possibilities of calcium binding soy proteins in various samples or products. Calcium binding of soy protein is an important factor to consider when assessing commercially available CFSM to ensure maximum calcium availability.

6.5 Conclusions

In summary, this study shows that the calcium bioavailability of marketed fortified soymilk could be assessed using the tracer method by labelling the fortificant after soymilk manufacture provided that a heat treatment was applied after the labelling process. The fluorescent image analyser and the gel sampling method on the electrophoresis gel samples of the labelled CFSM could not identify any binding of ^{45}Ca to the identified soy protein including: α'β-conglycinin, αβ-conglycinin and β portion-glycinin. However, it is recommended that further studies are undertaken to analyse the calcium binding properties of the soy proteins in fortified soymilk available commercially. The future studies would enhance the potential of formulating fortificants that could maximise calcium bioavailability.

Table 6.1 – Percentage of ^{40}Ca and ^{45}Ca isotope in the supernatant fraction of the calcium fortified soymilk.

Percent in supernatant	0 h						12 h						24h					
	Ca-40		Ca-45		Difference†		Ca-40		Ca-45		Difference†		Ca-40		Ca-45		Difference†	
Fortificant labelling:	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
before soymilk manufacture	21.4	2.4	17.2^{1}	0.5	4.2^{aA}	1.8	21.4	2.4	15.9^{1}	3.4	$5.5^{a,A}$	3.3	21.4	2.4	15.9^{1}	3.4	$5.5^{a,A}$	3.3
After soymilk manufacture – No heat treatment	21.8	3.1	44.0^{2}	3.1	22.1^{bB}	5.0	22.1	3.1	45.7^{2}	2.5	$23.6^{b,B}$	5.2	21.4	3.0	47.3^{2}	7.2	$25.9^{b,B}$	8.2
After soymilk manufacture – Heat treatment 90°C for 30 min	21.3	3.0	28.1^{3}	1.3	6.8^{aA}	4.1	22.0	3.1	28.8^{3}	5.2	$6.9^{a,A}$	5.1	22.0	3.1	25.0^{1}	3.7	$4.0^{a,A}$	1.3

† percent carrier (Ca^{40}) minus percent tracer (Ca^{45}) in the supernatant fraction. Data are given as mean values with their standard deviation

Mean percentages or mean differences in percentage in the same row, that have uppercase alphabets are significantly different ($P < 0.05$). Univariate analysis of variance used to compare means over time. Mean percentages or mean differences in the same column with different lowercase numbers and alphabets are significantly different ($P < 0.05$). Univariate analysis of variance was used to compare means and differences between treatments.

Table 6.2 – Radioactivity (cpm/mg Gel) of soy proteins run on SDS PAGE gels and labelled with ^{45}Ca. Protein bands were excised from the gel and dissolved in either 6% or 30% H_2O_2

	Blank	α'β – conglycinin (7S)	α-β – conglycinin (7S)	β portion – glycinin (7S)
6% H_2O_2 (cpm/mg Gel)	2.42 ± 1.31 [a]	1.42 ± 0.66 [a]	1.97 ± 0.84 [a]	1.38 ± 0.52 [a]
30% H_2O_2 (cpm/mg Gel)	6.43 ± 4.17 [b]	4.42 ± 2.53 [b]	5.73 ± 0.78 [b]	3.27 ± 2.15 [b]

Values are displayed as mean ± SD. Mean values in the same column with uppercase alphabets are significantly different ($p < 0.05$). Mean percentages or mean differences in the same row with different lowercase alphabets are significantly different ($P < 0.05$). Univariate analysis of variance used to compare means

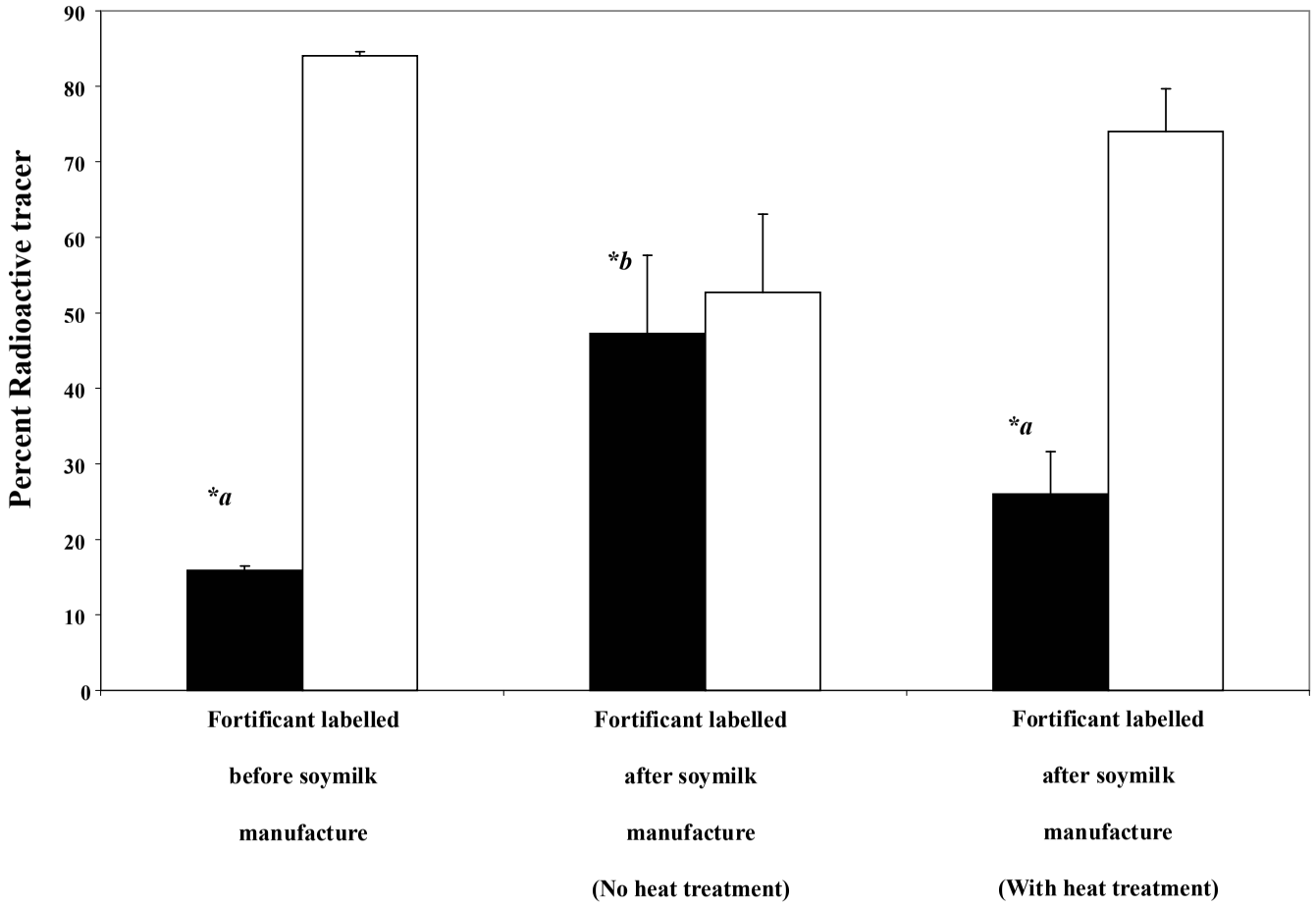

Figure 6.1 – Percentage of radioactive tracer in supernatant (solid bars) and residue portion (empty bars) of radiolabelled calcium fortified soymilk (CFSM) samples after 24 h equilibrium at 4°C with heat treatment of 90°C for 30 min.

(Mean values with different lower letters are significantly different $P < 0.05$)

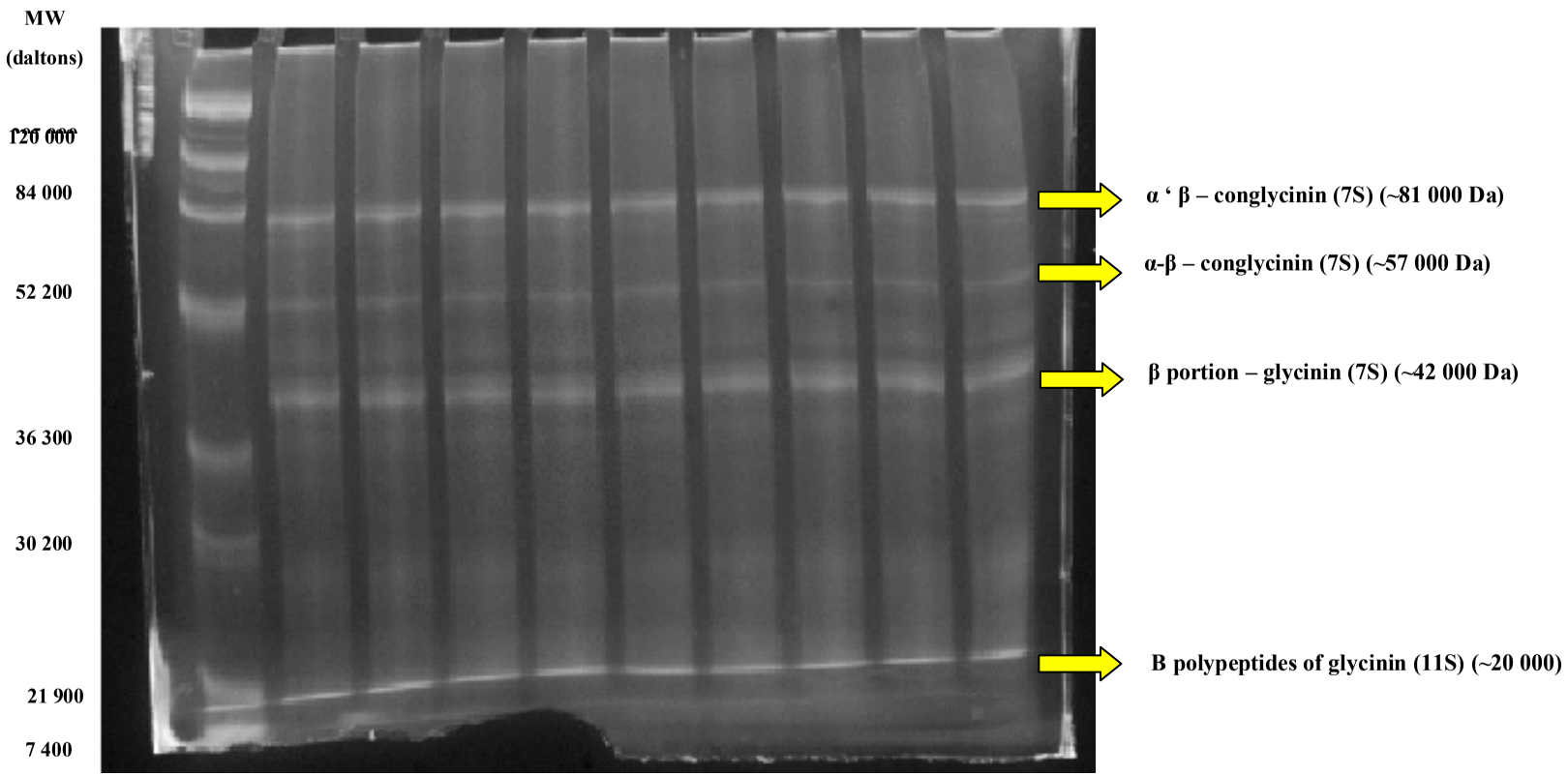

Figure 6.2 – Representative electrophoretic patterns of SDS-PAGE of soy protein in CFSM labelled after soymilk manufacture with ^{45}Ca.

[BR = broad range prestained molecular weight (MW) standards containing: myosin (MW 205,000), β-galactosidase (MW 120,000), bovine serum albumin (MW 84,000), ovalbumin (MW 52,000), carbonic anhydrase (MW 36,000), soybean trypsin inhibitor (MW 30,200), lysozyme (MW 21,900) and aprotinin (MW 7,400)].

Chapter 7
Calcium absorption in Australian osteopenic post menopausal women: An acute comparative study of fortified soymilk to cows' milk.**

7.1 Introduction

Calcium is a nutrient essential for maintenance of bone health and mineralisation (Nordin, 1997). In women, the loss of bone mineral greatly increases around the time of menopause as circulating oestrogen level declines. This in turn increases the risk of osteoporosis, a reduction in the amount of bone in the bone (Nordin, 2008). Prevention of osteoporosis in women depends in part on maintenance of a high calcium intake throughout life and particularly after menopause to slow the rate of bone loss ("Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society," 2010).

In Australia, around 60 percent of dietary calcium comes from dairy foods (*Calcium, Vitamin D and Osteoporosis - A guide for consumers*, 2008). Yet post menopausal women may choose to consume soymilk rather than cows' milk due to perceived health benefits such as reduced menopausal symptoms; although such effects

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still require substantiation (Setchell & Cassidy, 1999b). One negative effect from a change to soymilk may be reduced calcium intake as native soymilk contains much less calcium (~ 20mg/100mL) (Heaney, Dowell, Rafferty, & Bierman, 2000). Manufacturers have addressed this issue by fortifying soymilks with calcium, although methods of fortification vary considerably between products (Heaney, Rafferty, & Bierman, 2005).

Soymilk fortification appears to be an effective way to increase calcium intake and the total amount of absorbed calcium (Lopez - Huertas et al., 2006), although calcium bioavailability will depend considerably on the choice of fortificant (Zhao, Martin, & Weaver, 2005). To date, few studies have examined the absorption of calcium from different kinds of fortified soymilk available in the market (Heaney, Dowell, Rafferty, & Bierman, 2000; Zhao, Martin, & Weaver, 2005). To our knowledge, this is the first study examining calcium absorption from an Australian calcium fortified soymilk (CFSM).

7.2 Experimental methods

7.2.1 Calcium fortified soymilk

The calcium fortified soymilk (CFSM) used in this study is widely sold throughout Australia (*So Good*, *Sanitarium Health Foods, NSW, Australia*). It is made from soy protein (4%) and has been fortified to achieve similar calcium content to cows' milk (120mg/100 mL).

7.2.2 Participants and human study protocol

Twelve osteopenic, but otherwise healthy, post menopausal women aged between 50-65 yrs were recruited through poster, email and web advertisements. Potential participants were screened by telephone interview, and if the inclusion criteria were met, they were asked to complete a questionnaire to assess their health and menopausal status.

Women were included in the study if they were post menopausal (at least 12 months amenorrhea); had a diagnosis of osteopenia (i.e. with bone mineral density (BMD) T-score between -1 and -2.5 as measured by dual-energy X ray absorptiometry, Clinical Nutrition and Metabolism Unit, Monash Medical Centre, Clayton, Vic, Australia); otherwise generally healthy (no chronic disease by self-report, including gastrointestinal, kidney, liver, parathyroid or cardiovascular disease); not taking a medication or antibiotics that might affect calcium absorption; and not on hormone replacement therapy (HRT) during the preceding 12 months. In addition, participants were required to be lactose tolerant and not allergic to soy or soy products. Each participant completed an eating habit questionnaire (extracted from a food frequency questionnaire developed by the Australian Cancer Council, Vic, Australia) to assess their dietary calcium intake.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000), and all procedures involving human subjects were approved by the Southern Health Human Research and Ethics Committee. Written informed consent was obtained from all subjects.

7.2.3 Test milk drinks for the acute study

Milk drinks (cows' milk or CFSM) for the acute *in vivo* study were prepared by labelling the fortificant after CFSM manufacture as per described in chapter 6. In Brief, one microgram of $^{45}CaCl_2$ (Amersham Biosciences) was added in 44 mg calcium carrier to each 20 mL serve of milk giving a tracer concentration of 185 kBq/dose resulting in a specific activity of 4.2 kBq/mg of calcium. Milk samples were heat treated (90°C for 30 min) to ensure calcium equilibrium, then cooled and stored at 4°C for 24 h before use.

7.2.4 Study design

The participants were asked to come to Monash Medical Centre (MMC) to participate in a randomised crossover design calcium absorption study. Participants were tested on two separate occasions separated by a washout period of at least three weeks. At each test, subjects were randomised (with reference to a set of randomised numbers) to consume a radiolabelled milk sample, either cows' milk or CFSM. Participants arrived at MMC at 8.00 am, after an overnight fast. Bioelectrical impedance was determined (SFB7, Impedimed, Brisbane, Australia).

In the Department of Nuclear Medicine, a 22GA catheter was inserted in an antecubital vein of the participant to obtain a 10 mL baseline venous blood sample. Participants then consumed a 20 mL test sample of labelled CFSM or cows' milk, immediately followed by 200 mL of distilled water. Blood samples were then collected after 60 min following the method described by Nordin (Nordin, Morris, Horowitz, Coates, O'Loughlin, & Need, 2009; Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998).

Blood samples were centrifuged at 3,500 rpm for 10 min and the activity of the ^{45}Ca in the plasma aliquots (1 mL) was measured using a liquid scintillation counter (Wallac 1410) according to the method of Marshall and Nordin (Marshall & Nordin, 1981). The hourly fractional calcium absorption rate (α) was then calculated (Marshall & Nordin, 1981). Serum 25-hydroxy vitamin D was measured at the Pathology Department, Monash Medical Centre, Clayton, Vic, Australia using a commercially available kit (Diasorin, Saluggia, Italy).

7.3 Calculation and statistical analysis

The sample size of 12 women recruited into this crossover design study gives a probability of 90% that this human study can detect a treatment difference at a 5% level of significance (two-sided), if the true difference between the treatments is 15%. This is based on the assumption that the within-patient SD of the response variable is 10%. Data from this study were compared by Students' paired sample t-test. SPSS for Windows (version 11.5) was used for all statistical analyses. A *P* value of < 0.05 was taken as significant.

7.4 Results and discussion

7.4.1 Calcium absorption comparison of CFSM to cows' milk

Twelve post menopausal women with mean age (± SD) 56.7 ± 5.3 years were recruited for the calcium absorption study. They were mildly overweight (mean BMI 26.5 ± 5.6 kg/m^2).The body mass index (BMI) of women enrolled in the study ranged between 19-32 kg/m^{2}and they had serum 25-hydroxy vitamin D levels that were sub-

optimal to optimal, i.e. the mean value was 61.6 ± 18.9 nmol/L, 25% of the women had suboptimal values (< 50 nmol/L).

Table 7.1 shows the individual hourly fractional calcium absorption rates when the participants consumed either labelled CFSM or cows' milk. The mean hourly fractional calcium absorption rate (α) for each milk differed by only 1.5%, a difference that was not of statistical significance ($P > 0.05$).

7.4.2 Relation between serum 25-hydroxy vitamin D and calcium absorption

No relationship was evident between fractional calcium absorption and the serum 25-hydroxy vitamin D levels in study participants for either soymilk ($R^2 = 0.003$) or for cows' milk ($R^2 = 0.061$) (See Figures 7.1 and 7.2 respectively).

7.4.3 Discussion

Recruitment of the participants was very difficult due to the tight inclusion criteria. There were 90 women who enquired about the study and only 12 were able to complete the study due to factors such as: availability to come to the study, being comfortable with the minor exposure to the radioisotope and satisfy the inclusion criteria such as being osteopenic, not taking HRT, not on medication and more (as outlined in section 7.2.2). Figure 7.3 shows the type of milk regularly consumed by the osteopenic post menopausal women who participated in the study. Most of the women consumed a type of low fat milk, only 2 consumed full cream milk and only 1 frequently consumed soymilk. None of the women were smokers and most consumed a moderate amount of coffee per day (< 2 cups/day). Most of the women had a balanced

diet and they had no eating habits that could adversely affect calcium absorption. Figure 7.4 shows the number of participants out of 12 taking supplements (multivitamins, vitamin A, vitamin C, vitamin E, calcium, vitamin D, fish oils, cod liver oil, wheat bran, oat bran and fibre) as part of their usual diet. The most common supplements were calcium, vitamin D and fish oils.

In our study, serum 25-hydroxy vitamin D, which is the best indicator of vitamin D status, appeared unrelated to patterns of calcium absorption in the women studied. A review (Bronner, 2009) states that vitamin D does not play a significant role in increasing active calcium absorption when calcium intake is adequate.

Calcium absorption is controlled by calcitriol and not by calcidiol, so no relationship with calcium absorption would be expected. The relation between 25-hydroxy vitamin D and calcium absorption is entirely accounted for by the relation between 25-hydroxy vitamin D and $1,25(OH)_2$ vitamin D, the latter being the main determinant of calcium absorption. There is no evidence that 25-hydroxy vitamin D in its own right at physiological levels has any effect on calcium absorption.

Bioavailability studies require appropriate distribution of label in the test food. Tracer methods to measure calcium absorption are not commonly used on marketed products because they often cannot be easily labelled (Heaney, 2003). In chapter 6, we have shown that provided that a heat treatment is applied, a calcium fortificant can be labelled for tracer studies after it has been added to soymilk. This method then generates a tracer abundance very similar to that obtained when the fortificant has been labelled prior to its addition to soymilk. This heat treatment method was therefore used in this *in vivo* bioavailability study.

This study indicates that, in osteopenic post menopausal women who are at risk of developing osteoporosis, the bioavailability of calcium in CFSM is similar to that of cows' milk. Many factors can affect calcium absorption including protein content and type and individual absorptive patterns influenced by factors such as vitamin D (Kruger & Horrobin, 1997).

In previous studies, calcium absorption has been measured by a variety of methods;(Abrams, 1999; Abrams, Yergey, & Heaney, 1994; Bronner, 1998; Heaney, 2001; Heaney, Dowell, & Wolf, 2002; Moser-Veillon, Mangels, Vieira, Yergey, Patterson, Hill, & Veillon, 2001; Nickel, Berdine, Smith, Smith, Miller, & Weaver, 1996). The most common methods involve tracer methods employing stable or radioactive calcium isotopes (Hambridge, Krebs, Westcott, Sian, Miller, Peterson, & Raboy, 2005; Heaney, 2001; Schroder, Griffin, Specker, & Adrams, 2005). In this study, a single radioisotope test (Marshall, 1976) was selected as it provides fast and accurate results after the test dose is taken. This method is adopted as a valid basis for calculating calcium absorption (Nordin, Morris, Horowitz, Coates, O'Loughlin, & Need, 2009). The rate of calcium absorption calculated using this method correlated significantly with the rate of dietary calcium absorption measured in simultaneous calcium balances (Nordin, 1976) and very highly with calcium absorption calculated by the double isotope method (Nordin, Morris, Horowitz, Coates, O'Loughlin, & Need, 2009). The single isotope test produces a relatively sharp peak of radioactivity after 60 min and can therefore be completed over a short period of time (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998).

As calcium load increases absorption time and reduces test sensitivity (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998), a small (44 mg) calcium load was chosen for the present study. If the calcium load had been greater than 50 mg,

the double isotope method would have had to be used. The larger the carrier dose, the more interference occurs during the calcium absorption diffusion process and the less valid is the single isotope procedure (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998). A low dose has the advantage in that it appears to maximise differences between low calcium absorbers and high calcium absorbers (Wilkinson, 1976).

A validation study showed that the radioactivity in the plasma 60 min after administration of the test dose correlated so highly with the absorption rate calculated from the six blood sample that this single blood sample provided a satisfactory measure of calcium absorption (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998).

Calcium bioavailability is highly determined by the type of fortificant used (Heaney, Rafferty, Dowell, & Bierman, 2005). A recent study found that calcium fortificants added to beverages sold in the US had very different settling properties (Heaney, Rafferty, & Bierman, 2005). While some formed stable suspensions, others settled quickly on the bottom of the container (Heaney, Rafferty, & Bierman, 2005). A new calcium-enriched orange beverage had similar calcium absorption to that of cows' milk (Gonnelli, Campagna, Montagnani, Caffarelli, Cadirni, Giorgi, & Nuti, 2007). In soymilks, fortification with a type of calcium carbonate fortificant appears to yield similar calcium absorption to that of cows' milk, whereas CFSM fortified with tricalcium phosphate often has lower calcium absorption (Heaney, Dowell, Rafferty, & Bierman, 2000; Zhao, Martin, & Weaver, 2005). In the present study, the calcium fortificant in the CFSM used is a proprietary form of phosphate of calcium. The fractional calcium absorption from CFSM with this particular fortificant was equivalent to the fractional calcium absorption from normal cows' milk.

Apart from type of fortificant, calcium absorption may also be affected by the calcium/phosphorus ratio in foods, a determinant factor for mineral absorption and deposition into bone (Shapiro & Heaney, 2003). High levels of phosphate may precipitate calcium in the intestine rendering it unavailable (Schroder, Griffin, Specker, & Adrams, 2005). Differences in the calcium/phosphate ratio may thus potentially cause differences in calcium bioavailability between soy drinks apparently fortified with similar amounts of calcium. In the present study, this factor has been minimised since the CFSM used in this study appears to have a calcium/phosphorus ratio similar to that of cows' milk (0.8:1).

Phytic acid, which is present in many legumes including soybeans, is known to contribute to the poor bioavailability of many minerals, including calcium (Charoenkiatkul, Kriengsinyos, Tuntipopipat, Suthutvoravut, & Weaver, 2008; Messina, 1999). Soymilk has minimal amounts of phytic acid compared to other soy foods including soy flour, soy protein, soy nuts and textured vegetable protein (Al-Walsh, Horner, Palmer, Reddy, & Massey, 2005). This is particularly true for the soymilk used in this study, which is manufactured from isolated soy protein rather than from whole soybeans. The CFSM used has a phytic acid content of less than <0.1%, which is unlikely to impact on calcium absorption (Ashton, 2009) (unpublished results).

Soy is the major source of dietary phytoestrogen in the form of isoflavones (Adlercreutz et al., 1995) which has the potential to relieve some post menopausal symptoms (Jefferson, 2005) and may also reduce urinary calcium excretion, thereby potentially minimising bone loss (Setchell & Lydeking-Olsen, 2003a). Many epidemiological and some clinical studies have suggested that dietary phytoestrogens may also be helpful with respect to menopausal symptoms such as hot flushes (Murkies, Wilcox, & Davis, 1998). Isoflavone content however, appears to have little effect on

calcium bioavailability (Cai, Zhao, Glasier, Cullen, Barnes, Turner, Wastney, & Weaver, 2005; Spence, Lipscomb, Cadogan, Martin, Wastney, Peacock, & Weaver, 2005).

Some variability on fractional calcium absorption was observed on an individual basis, with 50% of the women having higher absorption from the CFSM than from cows' milk. Being an equol producer may positively help the optimum absorption of calcium from the CFSM compared to cows' milk. It has been shown that bone mineral density was increased during the consumption of fortified soymilk in women who were equol producers (Setchell, Brown, & Lydeking-Olsen, 2002).

While these findings indicate that calcium absorption was similar in this Australian CFSM and in cows' milk, investigations are needed over a longer term to show whether similar benefit accrues in bone building and maintenance. Moreover, further studies are needed to ascertain whether these benefits extend to other brands of soymilk supplemented with calcium.

7.5 Conclusion

The calcium absorption from CFSM fortified with a proprietary phosphate of calcium was found to be similar to that of cows' milk in osteopenic post menopausal women. Soymilk can therefore be successfully fortified to deliver the same levels of calcium as cows' milk, and can thus be confidently used as a substitute for cows' milk in the diets of post menopausal women, vegetarians and individuals with lactose or other forms of dairy food intolerance.

Table 7.1 – Hourly fractional calcium absorption rate (α) of cows' milk and fortified soymilk in 12 osteopenic post menopausal women.

	Hourly fractional absorption rate (α)	
Participant Code	**Cows' Milk**	**Soymilk (CFSM)**
101	0.77	0.72
102	0.62	0.90
103	0.72	1.00
104	0.76	0.94
105	0.51	0.64
106	0.68	0.67
107	0.90	0.53
108	0.68	0.74
109	0.55	0.67
110	0.38	0.37
111	0.93	0.43
112	0.32	0.31
Mean ± SD	**0.66 ± 0.22**[a]	**0.65 ± 0.19**[a]

[a] no significant difference ($P < 0.05$) between means. Data given are mean values with their standard deviation

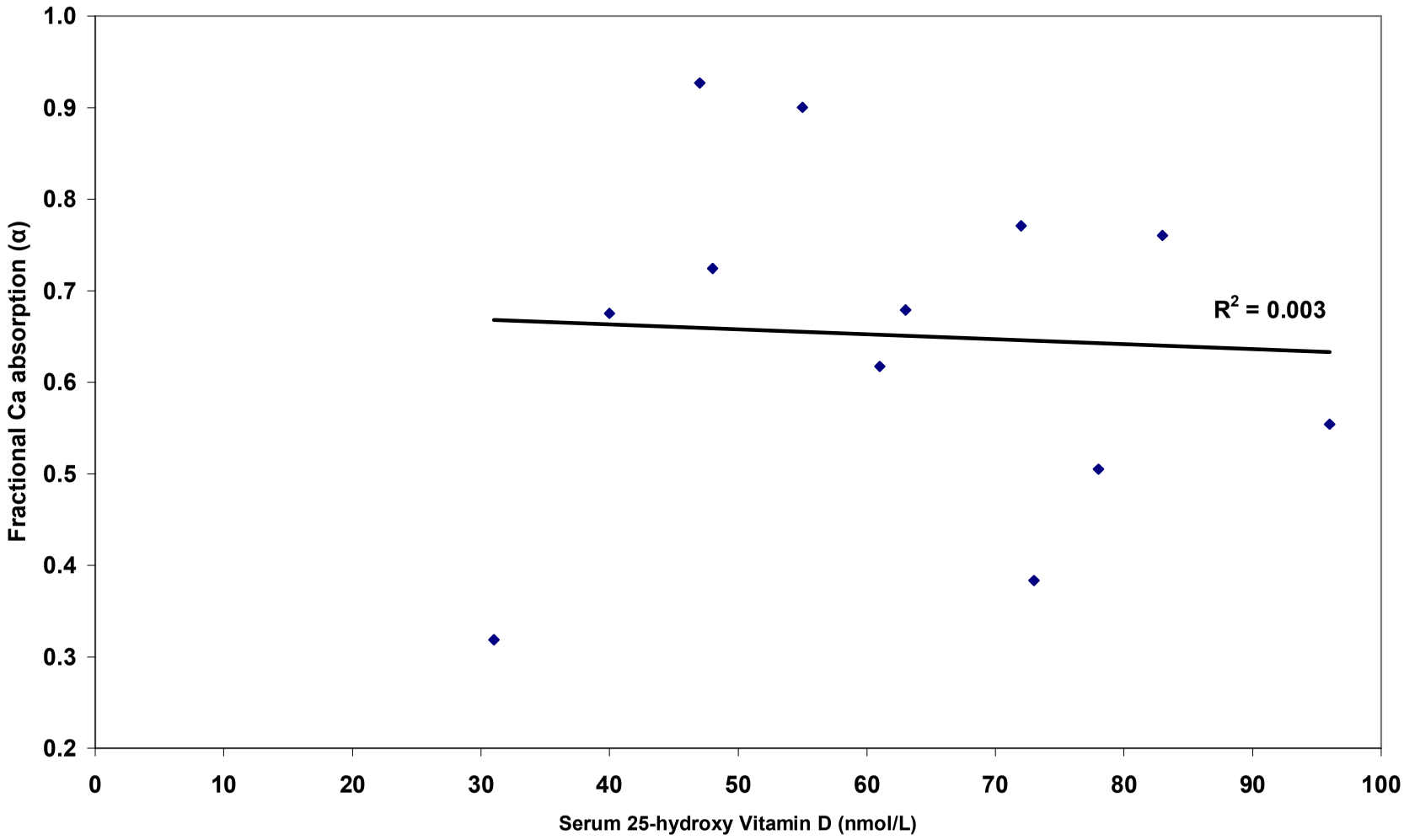

Figure 7.1 – Effect of serum 25-hydroxy vitamin D on fractional calcium absorption (α) from CFSM

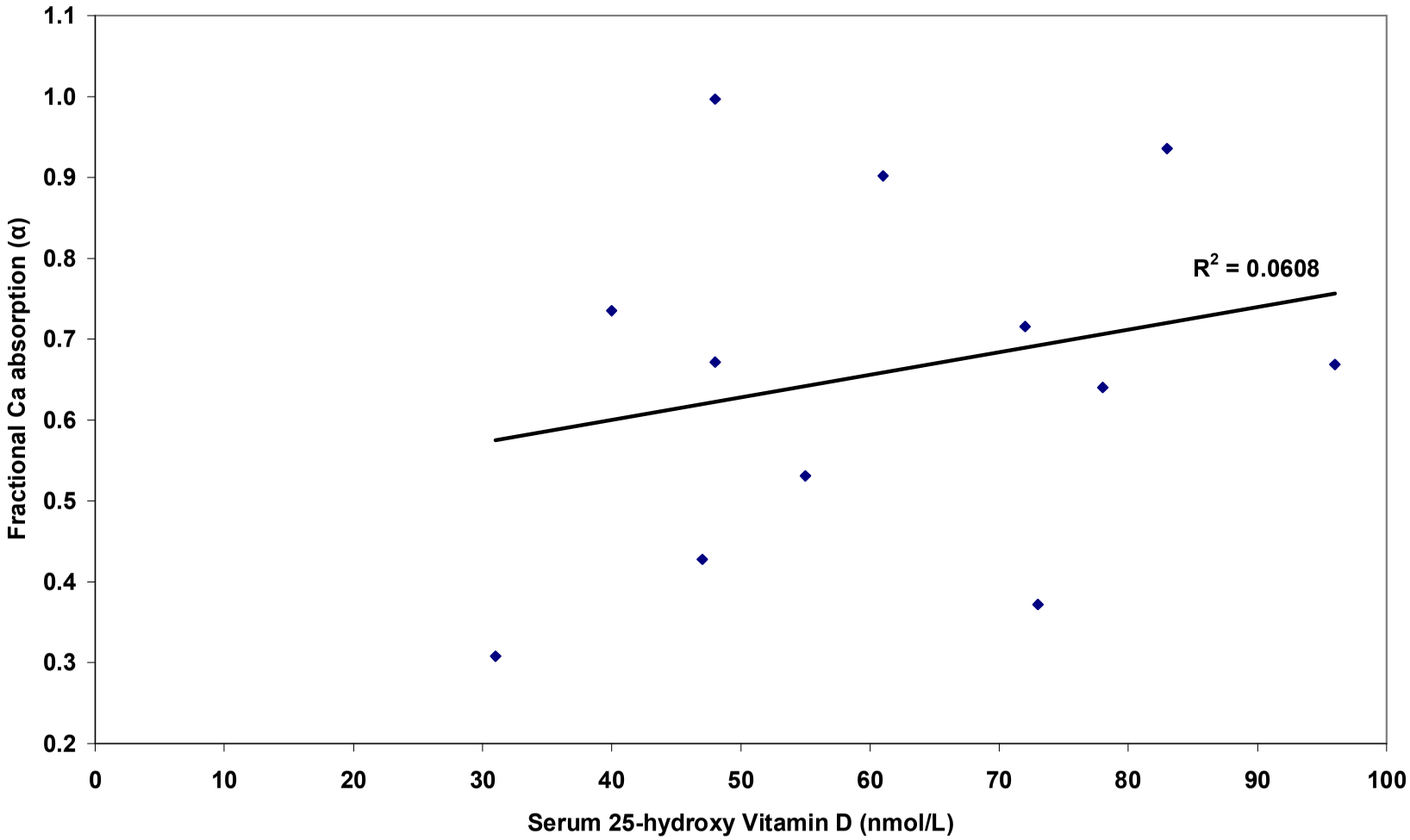

Figure 7.2 – Effect of serum 25-hydroxy vitamin D on fractional calcium absorption (α) from cows' milk

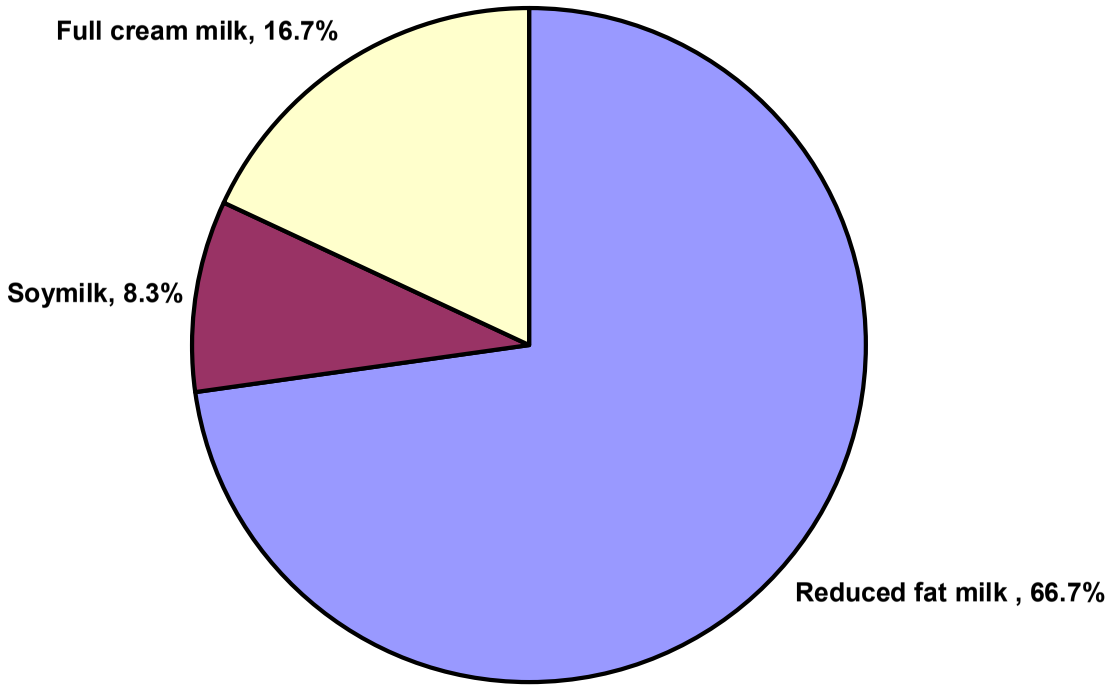

Figure 7.3 – Types of milk consumption by post menopausal women who participated in the study

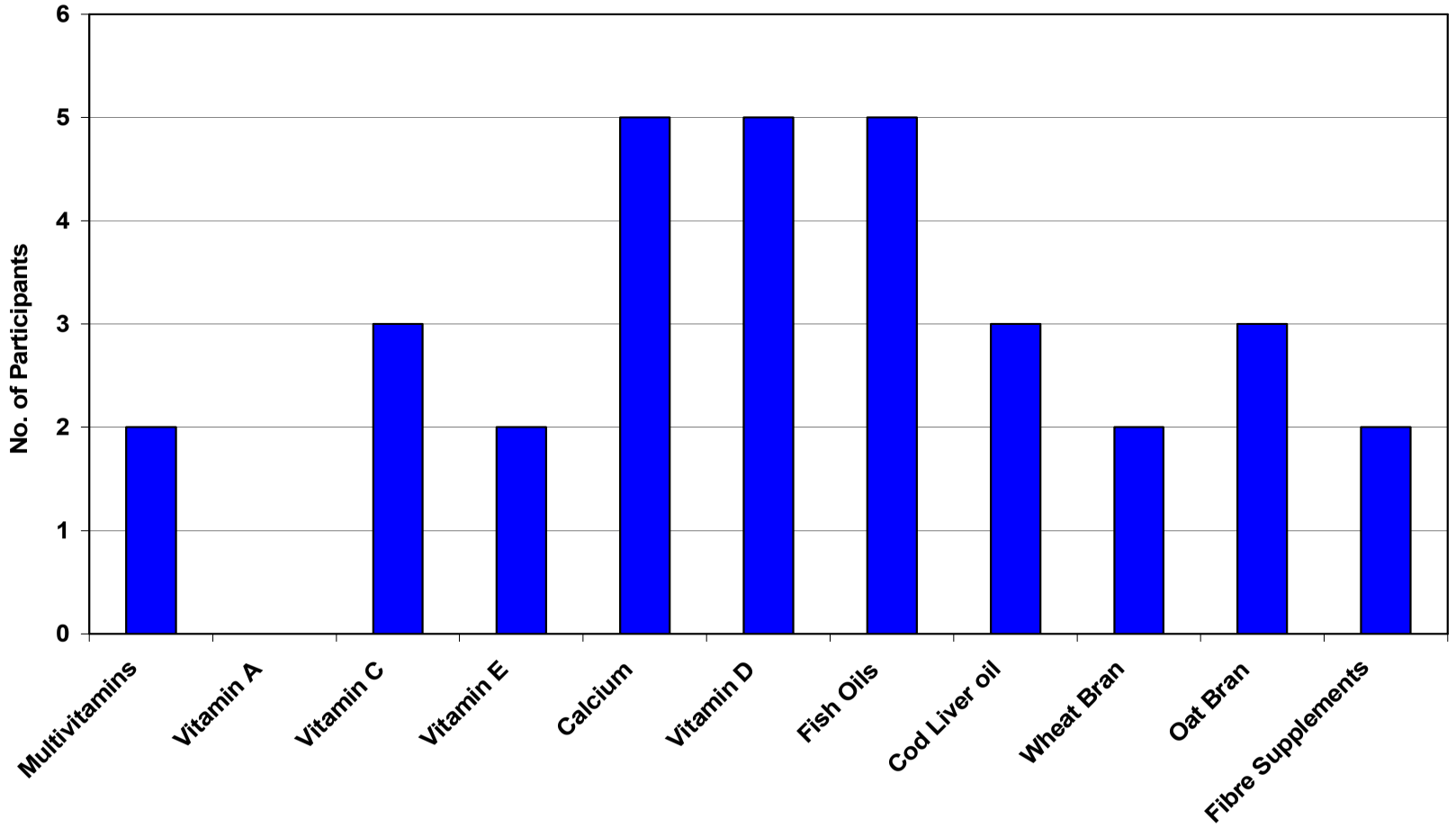

Figure 7.4 – Number of participants out of 12 taking multivitamin, Vitamin A, Vitamin C, Vitamin E, Calcium, Vitamin D, Fish Oils, Cod Liver Oil, Wheat Bran, Oat Bran and Fibre Supplements as part of their usual diet

Chapter 8
Equol production in Australian osteopenic post menopausal women from study

8.1 Introduction

Recent studies have emphasised 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 daidzein and genistein because it has a structure similar to human 17β-estradiol (Adlercreutz et al., 1995). Equol is suggested to be more oestrogenically active than daidzein, having a greater affinity for estrogen receptors (ERs) (Morito et al., 2001). Following soy consumption, equol is bacterially derived in the gut from daidzin. Unlike soy isoflavones, daidzein or genistein, equol has a chiral centre and can occur as two distinct diastereoisomers (Setchell et al., 2005). A study with rats showed that equol is more than twice as oestrogenic as genistein (Setchell, Brown, & Lydeking-Olsen, 2002). 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 (Setchell & Lydeking-Olsen, 2003a; Ward, Kim, Chan, & Fonseca, 2005). The ability to produce equol appears to depend on the composition and enzymatic capability of the gut microflora (Setchell, Brown, & Lydeking-Olsen, 2002). Equol has the greatest antioxidant activity of all the isoflavones tested when *in vitro* measured and this may provide greater inhibition of lipid peroxidation and therefore greater reduction in risk

for cardiovascular disease (Setchell, Brown, & Lydeking-Olsen, 2002; Wijeratne & Cuppett, 2007). Several recent studies suggest that those who are equol producers show more favourable responses to soy isoflavone-containing diets (Setchell, Brown, & Lydeking-Olsen, 2002), indicating that the metabolite S-equol has greater biological activity than either daidzein or genistein (Setchell & Cole, 2006).

In Chapter 7, it was reported that calcium absorption from calcium fortified soymilk was equivalent to the calcium absorption from cows' milk. Out of 12 women, 6 of them (50%) absorbed calcium from fortified soymilk better than calcium from cows' milk. To date, no studies have demonstrated whether equol producers may exert better calcium absorption from calcium fortified soymilk compared to cows' milk.

The objective of the study was to determine the equol status of the participants to investigate why 50% of the participants' fractional calcium absorption (α) value was higher from fortified soymilk that cows' milk. The aim was also to observe whether being an equol producer may have a higher fractional calcium absorption value compared to none equol producers.

8.2 Experimental methods

8.2.1 Participants and human study protocol

Twelve osteopenic, but otherwise healthy, post menopausal women aged between 50-65 yrs who participated in the previous study (Chapter 7) accepted to participate in determining if they were equol producers. Participants had no pre-existing gastrointestinal and liver disease. No dietary restrictions were imposed and the participants continued to consume their usual diets. This study was conducted according to the guidelines laid down in the Declaration of Helsinki in 1995 (as revised in

Edinburgh in 2000) and all procedures involving human subjects were approved by the Health, Engineering and Science Human Research Ethics Committee at Victoria University, Melbourne. Written informed consent was obtained from all subjects.

8.2.2 Calcium fortified soymilk

The calcium fortified soymilk (CFSM) used in this study is widely available throughout Australia (*So Good, Sanitarium Health Foods, NSW, Australia*). It is made from soy protein (4%) and has been fortified to achieve similar calcium content to cows' milk (120mg/100mL).

8.2.3 Test milk

On three consecutive days, each participant was given 2 glasses (250 mL) of the calcium fortified soymilk, 1 in the morning and 1 in the evening. On the morning of Day 3, each subject voided their bladder and began collection of a pooled 24-h urine sample that was stored at 4°C during the collection period. The 24-h urine volume was recorded and 50 mL of the urine was frozen until analysis. Urine samples were sent for analysis.

8.2.4 Analytical methodology

S-equol, daidzein and genistein concentration were measured from urine by HPLC with electrospray ionisation (ESI)-MSI (Setchell, Brown, Desai, Zimmer-Nechemias, Wolfe, Brashear, Kirschner, Cassidy, & Heubi, 2001b; Setchell & Cole, 2006; Setchell et al., 2003; Setchell, Maynard Brown, Desai, Zimmer-Nechemias,

Wolfe, Jakate, Creutzinger, & Heubi, 2003) by the Division of Pathology, Cincinnati Children's Hospital Medical Centre, Cincinnati, in collaboration with Prof. Kenneth D R Setchell, and Dr. John Ashton, from Sanitarium Development and Innovation, Cooranboong, NSW. The analyses were performed by the above collaborators and using the data, a clear distinction was determined between equol producers and non equol producers from the urinary samples.

8.3 Data analysis

The samples were analysed in duplicates. The average value of equol to daidzein ratio determined whether participants were equol producers.

8.4 Results and discussion

8.4.1 Results

Table 8.1 shows the hourly fractional calcium absorption rate of cows' milk and fortified soymilk in 12 osteopenic post menopausal women as well as their equol production status. From our results, only one participant was classified as a definite 'equol producer'. The fractional calcium absorption of that participant (code 107) from calcium fortified soymilk was not greater than cows' milk ($\alpha = 0.53$ and $\alpha = 0.90$ respectively). This analysis therefore could not determine whether equol producers can absorb more calcium from fortified soymilk than from cows' milk.

Table 8.1 – Hourly fractional calcium absorption rate (α) of cows' milk and fortified soymilk in 12 osteopenic post menopausal women and their respective equol producing status

	Hourly fractional absorption rate (α)		
Participant Code	**Cows' Milk**	**Soymilk (CFSM)**	**Equol Producers (Yes or No)**
101	0.77	0.72	No
102	0.62	0.90	No
103	0.72	1.00	No
104	0.76	0.94	No
105	0.51	0.64	No
106	0.68	0.67	No
107	0.90	0.53	Yes
108	0.68	0.74	No
109	0.55	0.67	No
110	0.38	0.37	No
111	0.93	0.43	No
112	0.32	0.31	No
Mean ± SD	**0.66 ± 0.22**[a]	**0.65 ± 0.19**[a]	

[a] no significant difference ($P < 0.05$) between means. Data given are mean values with their standard deviation.

8.4.2 Discussion

This experiment was a pilot/preliminary exercise. The results show that only about 9% of the participants are equol producers compared to the claimed 20-35% of the general adult population (Setchell et al., 2005). This analysis was determined using a very small sample size (n = 12), and is not therefore a sufficient representation of the general adult population. This exercise could not show any correlation between enhanced calcium absorption from food sources or fortified soymilk source for equol producers. Previous studies have shown the beneficial role of soy isoflavones, including equol, in preventing osteoporosis due to their bone sparing effects (Chen, Ho, Lam, Ho, & Woo, 2003a; Rufer, Bub, Moseneder, Winterhalter, Sturtz, & Kulling, 2008; Setchell & Lydeking-Olsen, 2003a) during soy isoflavone consumption. The exact mechanisms of action are presently elusive or speculative (Setchell & Lydeking-Olsen, 2003a). A study on Chinese adolescent girls aged 14 to 16 showed that 375 ml of calcium fortified soymilk, or the equivalent of about two glasses, is among the effective strategies for bone acquisition and the optimisation of peak bone mass in adolescent girls (Ho, Chan, Yi, Wong, & Leung, 2001). Another study in ovariectomised rats showed that long term equol consumption like genistein and daidzein provide bone sparing effects, and indigestible sugars or probiotics such as *L. casei* in the diet significantly improves the protective effect on the skeleton (Mathey et al., 2007). Although the mechanisms of isoflavones are still not completely known, evidence from in vitro studies suggests that they act in multiple ways, via genomic and nongenomic pathways and via both osteoblasts and osteoclasts, to maintain bone mass (Atmaca, Kleerekoper, Bayraktar, & Kucuk, 2008).

A study compared soy protein isolate with either casein or 17β-estradiol and found that soy protein improved BMD by 15% compared with control and was as effective as estradiol (Arjmandi, Alekel, & Hollis, 1996). However, they could not demonstrate whether this improvement was due to the protein itself or the presence of soy isoflavones. Isoflavone content made the difference in improving BMD, and the bone-sparing effect might be due to increased intestinal calcium absorption (Arjmandi, Birnbaum, & Goyal, 1998) Another study reported that genistein prevented both trabecular and cortical bone loss in ovariectomized rats. Genistein increased bone formation, osteoblast number, and osteocalcin level and blocked the increased production of TNF-β, which might be responsible for bone-sparing effects of genistein (Fanti, Monier-Faugere, & Geng, 1998). Long-term studies also indicate that soy-protein intake protects kidneys, whereas excessive animal-protein intake may be harmful to kidneys. In animal models of kidney disease, rats fed soy protein had much slower progression of renal disease than did rats fed casein (Anderson, Smith, & Washnock, 1999).

The mechanism of action for the apparent bone sparing effect from the consumption of soy isoflavone from being an equol producer is not known. Many studies have demonstrated the bone sparing effects of soy isoflavone consumption in the general population. Equol production status should be a part of the inclusion criteria while undertaking studies that examine the effect of soy isoflavone consumption on calcium absorption or bone retention. No previous studies have researched the hypothesis whether being an equol producer may enhance fractional calcium absorption from soy sources. This pilot analysis would have helped determine one of the possible mechanisms of actions for bone sparing effect of soy isoflavone consumption; however,

sample size was the major limiting factor in this study. The concept is worth pursuing for future studies.

8.5 Conclusion

No correlation between equol production status and enhanced fractional calcium absorption values was observed, possibly due to the small sample size. A review of the literature indicates that equol producers may express higher bone sparing effects than non equol producers. Future studies involving larger sample sizes are required to determine if equol producers express higher fractional calcium absorption values that non equol producers from food sources especially soy sources.

Chapter 9

Calcium absorption in Australian osteopenic post menopausal women: An acute comparative study of fortified soymilk to fermented fortified soymilk[††]

9.1 Introduction

Soymilk is increasingly consumed in developed countries. It is rich in protein and is often recommended to women as a source of isoflavones. Potential benefits include relief from hot flushes, improved lipid profiles, protection against oxidative damage to DNA and in particular, maintenance of bone health (Atkinson, Compston, Day, Dowsett, & Bingham, 2004; Lydeking-Olsen, Beck-Jensen, Setchell, & Holm-Jensen, 2004; Setchell & Lydeking-Olsen, 2003a). Long term consumption of isoflavones can have bone-sparing effects due to attenuation of bone loss (Atkinson, Compston, Day, Dowsett, & Bingham, 2004; Chen, Ho, Lam, Ho, & Woo, 2003a).

Natural soymilk contains only about 20 mg/100 mL of calcium compared to cows' milk which contains about 120 mg/100 mL. Commercially available soymilk is

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now fortified to the same level as cows' milk by adding calcium phosphate, calcium carbonate or calcium chloride. Not all calcium fortificants, however, are equivalent (Heaney, Rafferty, & Bierman, 2005). Some may have poor solubility. The bioavailability rather than the total content of calcium in soymilk is thus an important issue. Calcium bioavailability is improved by the presence of high amounts of soluble calcium in food (Schroder, Griffin, Specker, & Adrams, 2005; Theobald, 2005) and by facilitating ionisation of calcium in the digestive system.

One way to potentially enhance the biological activity and nutritional value of soymilk is through fermentation with probiotics. In Chapter 4, we showed that fermentation of soymilk *in vitro* with β-glucosidase-producing probiotic bacterial strains allows acetyl- and β-glucoside isoflavones to undergo enzymatic hydrolysis into biologically available aglycone structures and also increases calcium solubility (Tang, Shah, Wilcox, Walker, & Stojanovska, 2007). Aglycones are absorbed faster and in greater amounts than their corresponding glucosides (Izumi, Piskula, Osawa, Obata, Tobe, Saito, Kataoka, Kubota, & Kikuchi, 2000; Kano, Takayanagi, Harada, Sawada, & Ishikawa, 2006).

In addition, probiotics are living microbial food supplements which may have beneficial effects on human health as stated in the previous chapters. Some of the benefits include: alleviation of symptoms of lactose intolerance, atopic disorders and coeliac disease, and are useful in the treatment of diarrhoea, ulcerative colitis and irritable bowel syndrome (Gibson, Rastall, & Fuller, 2003). They also confer protection against urogenital tract and *Helicobacter pylori* infections (Lionetti, Indrio, Pavone, Borrelli, Cavallo, & Francavilla, 2010), and there have been claims for cholesterol-lowering effects, anti-carcinogenic actions and improved immune function (Tannock, 2005).

Post menopausal women are at high risk of osteoporosis following increased bone loss during and after menopause. Fermentation with probiotics can provide various health benefits, however, little is known on whether fermentation of soymilk will also affect calcium absorption. In this study, we investigate whether fermentation of fortified soymilk improves hourly fractional calcium absorption. A well-established single blind crossover radioisotope method was used to compare calcium absorption from a calcium fortified soymilk versus a fermented calcium fortified soymilk in osteopenic but otherwise healthy Australian post menopausal women.

9.2 Experimental methods

9.2.1 Calcium fortification of soymilk

The calcium fortified soymilk (CFSM) used in this study is one widely sold throughout Australia (*So Good, Sanitarium Health Foods, NSW, Australia*). It is made from soy protein isolate (4%) and has been fortified with a proprietary phosphate of calcium to achieve similar calcium content to that of cows' milk (120 mg/100mL).

9.2.2 Labelling of the fortificant with ^{45}Ca after soymilk manufacture

The fortificant present in the CFSM was labelled by adding one microgram of high-specific-activity $^{45}CaCl_2$ to 20 mL amount of soymilk, yielding a tracer concentration of around 185kBq required for the calcium absorption test. Labelled CFSM was vortexed continuously for 1 min and then heat-treated (90°C for 30 min) before storing at 4°C for 24 h to allow for calcium exchange. This treatment was followed by fermentation with *L. acidophilus* ATCC4962 as required.

9.2.3 Bacteria

A pure culture of *Lactobacillus acidophilus* ATCC4962 was obtained from the Victoria University Culture Collection (Werribee, Victoria, Australia). Its purity was checked by gram staining before storage at -80ºC in 40% glycerol.

9.2.4 Fermentation of calcium fortified soymilk

The probiotic culture *L. acidophilus* ATCC4962 was activated through three successive transfers in MRS broth (De Mann, Rogosa, & Sharpe, 1960) at 37ºC for 20 h using a 2% inoculum. Labelled CFSM was then aseptically inoculated with a 1% (v/v) inoculum before incubation at 37ºC for 24 h. The fermented drink was then stored for a maximum period of 48 h at 4˚C until consumption.

9.2.5 Participants and human study protocol

Twelve osteopenic but otherwise healthy post menopausal women aged 50-68 yrs were recruited through advertisement using posters, e-mail and the internet. Potential participants were screened by telephone interview. If inclusion criteria were met, they were asked to complete a questionnaire to determine health and menopausal status. Women were included in the study if they were post menopausal (with amenorrhea for at least 12 months); had been diagnosed as osteopenic (i.e. had a bone mineral density (BMD) T-score between -1 and -2.5 as measured by dual-energy X-ray absorptiometry, Body Composition Laboratory, Clinical Nutrition and Metabolism Unit, Monash Medical Centre, Clayton, Vic, Australia); were otherwise generally healthy (no chronic disease by self-report, including gastrointestinal, kidney, liver, parathyroid or cardiovascular disease); were non-smokers; were not taking a medication or antibiotics

that might affect calcium absorption; and had not been on hormone replacement therapy (HRT) during the preceding 12 months. In addition, participants were required to be lactose tolerant and not allergic to soy or soy products.

The body mass index (BMI) of women participating in the study ranged between 19 to 36 kg/m^2. Each participant completed an eating habit questionnaire (extracted from a food frequency questionnaire developed by the Australian Cancer Council, Vic, Australia) to assess dietary calcium intake. This study was conducted according to the guidelines laid down in the Declaration of Helsinki in 1995 (as revised in Edinburgh in 2000) and all procedures involving human subjects were approved by the Southern Health Human Research and Ethics Committee (SHHREC). Written informed consent was obtained from all subjects.

9.2.6 Test milk drinks for the acute study

Soymilks (CFSM and fermented CFSM) for the acute study had a tracer concentration of 185 kBq/dose. Each dose comprised 20 mL of CFSM or fermented CFSM containing a microgram amount of $^{45}CaCl_2$ (Amersham Biosciences, Rydalmere, Australia) in a total of 44 mg of calcium carrier (20 mg as ^{45}Ca and 24 mg as ^{40}Ca present in the CFSM). 200 mL of distilled water was consumed immediately after ingestion of the test soymilk.

9.2.7 Study design

Participants were asked to come to Monash Medical Centre to participate in a randomised crossover design calcium absorption study. Participants were tested on two distinct occasions separated by a washout period of at least three weeks. For each test,

subjects were randomised (with reference to a set of randomised numbers) to consume a radio-labelled soymilk sample, either CFSM or fermented CFSM.

Participants arrived at the Clinical Nutrition and Metabolism Unit, at 8.00 am, after an overnight fast. Bioelectrical impedance was determined (SFB7, Impedimed, Brisbane, Australia). Participants then moved to the Department of Nuclear Medicine where a 22 GA catheter was inserted in an ante-cubical vein. A 10 mL venous blood sample was taken for the baseline and for the measurement of serum 25-hydroxy vitamin D. Participants then consumed the 20 mL test sample of CFSM or fermented CFSM, immediately followed by 200 mL of distilled water.

Blood samples were collected after 60 min following the method described by Nordin (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998). Blood samples were centrifuged at 3,500 × g for 10 min and the activity of the ^{45}Ca in 1 mL plasma aliquot was measured using a liquid scintillation counter (Wallac 1410, Perkin Elmer Life Sciences, Massachusetts, USA) according to the method of Marshall and Nordin (Marshall & Nordin, 1981). The fractional calcium absorption (α) was then calculated (Marshall & Nordin, 1981).

9.3 Calculation and statistical analysis

The sample size of 12 women recruited into this crossover pilot design study gives a probability of 90% that this human study can detect a treatment difference at a 5% level of significance (two-sided), if the true difference between the treatments is 15%. This is based on the assumption that the within-patient SD of the response variable is 10%. Data from this study were compared by Student's paired t-test. SPSS

for Windows (version 11.5) was used for all statistical analyses. A P value of < 0.05 was taken as significant.

9.4 Results and discussion

9.4.1 Comparison of calcium absorption from CFSM vs fermented CFSM

The mean age (±SD) of the 12 post menopausal women was 54.8 ± 12.3 years. They were mildly overweight (mean BMI 26.5 ± 5.5 kg/m^2). Mean serum 25-hydroxy vitamin D was 62.6 ± 19.1 nmol/L (range 31-96 nmol/L), and while most women had normal levels, 5 out of the 12 (42%) had vitamin D insufficiency (serum 25-hydroxy vitamin D < 50 nmol/L).

The mean fractional calcium absorption values for the study are shown in Table 9.1. The mean fractional calcium absorption (α) of the fermented CFSM was around 10% higher compared with that of unfermented CFSM, a difference that was not of statistical significance ($P = 0.122$). The individual differences in fractional calcium absorption between fortified soymilk and fermented fortified soymilk in the participants are shown in Figure 9.1.

9.4.2 Discussion

In vitro studies have indicated that the fermentation of soymilk with some probiotics may enhance calcium solubility and bioavailability (Tang, Shah, Wilcox, Walker, & Stojanovska, 2007; Tsangalis, Ashton, Stojanovska, Wilcox, & Shah, 2004). To date, no other studies have examined the effects of fermenting CFSM on calcium absorption in humans. In this study, participants were non-vegetarian and 77% rarely

consumed soymilk or soy products. 5 of the 12 participants regularly took calcium and vitamin D supplements. All performed only low to moderate physical activity. Habitual intake of calcium (by self report) was moderate and calcium supplementation was avoided during the study. Calcium intake by participants is thus unlikely to have affected our results; moreover, from the crossover design of the study, each participant acted as her own control.

The modified study method was based on the single isotope radiocalcium absorption test, a robust, well-validated measure of calcium absorption (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998). This modified method would also be applicable to other drinks fortified with calcium and is valuably used when undertaking comparative studies. The test can be completed over a short period of time, allowing absorption from a short segment of small intestine to be followed via a sharp peak of radioactivity (Nordin, 1976). The rate of calcium absorption measured by this method correlates strongly with that measured in balance studies and very highly with double isotope calcium absorption tests (Nordin, Morris, Horowitz, Coates, O'Loughlin, & Need, 2009). It is important however, when employing this method, to use a small calcium load (e.g. 44 mg as in the present study) as a higher calcium load will increase absorption time and reduce test sensitivity. The larger the carrier dose, the more interference occurs during the calcium absorption diffusion process and the less valid is the single isotope procedure (Nordin, Morris, Wishart, Scopacasa, Horowitz, Need, & Clifton, 1998).

The labelling of the fortificant with ^{45}Ca after soymilk manufacture was shown to have a tracer distribution pattern very similar to that when the fortificant was labelled before the soymilk manufacture, provided a heat treatment was applied (Tang, Walker, Wilcox, Strauss, Ashton, & Stojanovska, 2010). In this study, the soymilk fortificant

was also labelled prior to the fermentation. No studies have indicated negative effects of probiotics on the availability of the ^{45}Ca radioisotope during calcium absorption, although a recent study found that Ca^{2+} plays a catalytic role for human gut colonic bacteria (Zhu et al., 2010).

We have shown that fermenting CFSM with *L. acidophilus* ATCC4962 generated no apparent improvement in fractional calcium absorption in 12 osteopenic post menopausal women. The insignificant effect of fermentation on calcium bioavailability observed in the present study may in part reflect our choice of CFSM for fermentation. We have previously demonstrated that the fractional calcium absorption (α) from the CFSM used in this present study is comparable to that of cows' milk (Chapter 7). The fortificant present in this CFSM may already be in its most absorbable form so that fermentation in this case does not significantly improve acute calcium absorption. Optimum calcium absorption (α) was observed one hour after ingestion of the unfermented soymilk. It remains possible that fermentation will improve calcium absorption in other soymilks where other methods of calcium fortification have been used. It would thus be valuable to compare calcium bioavailability with and without fermentation for several different types of commercially available fortified soymilk.

Even without any change in calcium bioavailability, fermentation may have nutritional benefits as it significantly increases aglycone content, i.e. increased daidzein, glycetein and genistein (Tang, Shah, Wilcox, Walker, & Stojanovska, 2007). Fermentation also increases the solubility of calcium by decreasing the pH of CFSM. Moreover, the phytase enzyme produced by some probiotics will hydrolyse phytic acid and IP6, generating myo-inositols with reduced numbers of phosphate groups (IP3–IP5) (Haros, Bielecka, & Sanz, 2005; Haros, Carlsson, Almgren, Larsson-Alminger, Sandberg, & Andlid, 2009), which then has beneficial effects on calcium

bioavailability. In the present study, however, the CFSM used was made from soy protein isolate rather than a whole soy bean and even prior to fermentation, had minimal phytic acid content.

Figure 9.1 shows the individual fractional calcium absorption from CFSM to fermented CFSM. 4 out of the 12 post menopausal women absorbed calcium better from the fermented CFSM than from the non fermented CFSM. This observation may be correlated with another known calcium absorption facilitating mechanism, which are 'equol producers' (Setchell & Cole, 2006). These women may have come from the approximate one third of the population who are such 'equol producers'. In further studies, it would be advised to assess equol producing status via a 24-hour urine excretion (Setchell & Cole, 2006).

Although this acute study suggests that fermentation of CFSM has no effect on calcium bioavailability, the conditions of the study examined only acute absorption of calcium from the small intestine. It remains possible that fermented CFSM may facilitate slower calcium absorption from the large intestine. Further longer term studies with larger numbers of women would be valuable. *Post hoc* analysis of these results suggests that for adequate statistical power, 174 subjects would be needed per treatment group. Long term consumption of fermented CFSM could also provide the groundwork for studies of bone density measurements. Our pilot study may thus have been underpowered to detect any small difference in bioavailability between the two test drinks. It does not exclude a greater difference with fermentation in different soymilks fortified by other methods. Our findings do not therefore preclude possible benefits on long term consumption of fermented CFSM on bone health and calcium balance.

9.5 Conclusion

In summary, during this acute pilot study, there was no significant improvement on fractional calcium absorption from the ingestion of non fermented CFSM and fermented CFSM with *L. acidophilus* ATCC4962 in osteopenic post menopausal women. To observe a significant improvement on fractional calcium absorption, a larger sample size study would be required. Longer studies would be required to determine whether fermentation of CFSM may also contribute to the potential cumulative long term benefits on calcium bioavailability and bone health.

Table 9.1 – Mean fractional calcium absorption (α) of fermented calcium fortified soymilk and fortified soymilk in 12 osteopenic post menopausal women.

	Fractional absorption (α)	
	Fermented and calcium fortified soymilk	**Calcium fortified soymilk**
Mean (α) ± SD	**0.71 ± 0.29** [a]	**0.64 ± 0.23** [a]

[a] no significant difference ($P = 0.122$) between means. Data given are the mean ± SD

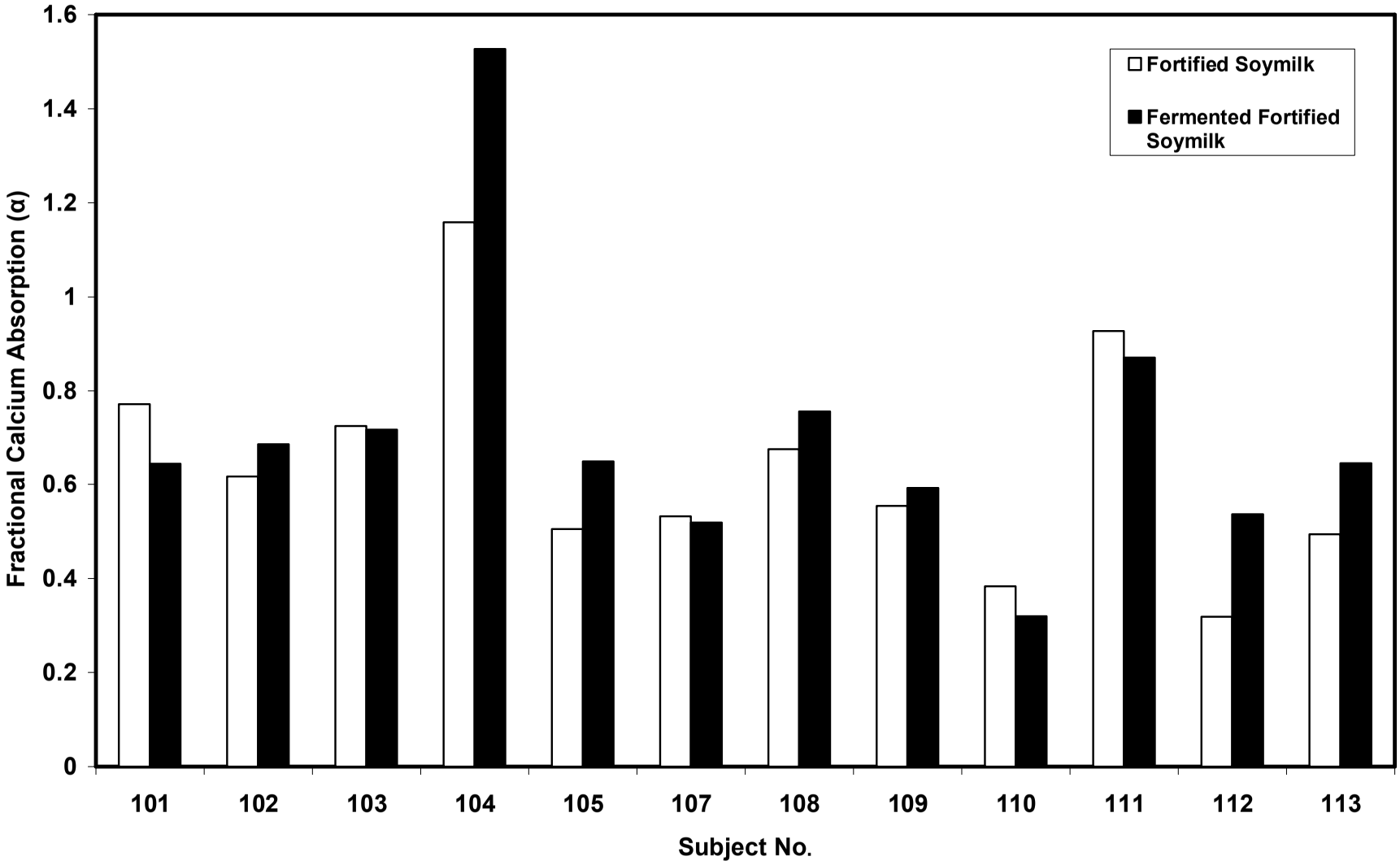

Figure 9.1 – Fractional calcium absorption (α) of fortified soymilk and fermented fortified soymilk of participants

Chapter 10
Overall conclusions and future research

10.1 Overall conclusions

The *in vitro* studies showed that the viability of the selected LAB strains during fermentation of calcium fortified soymilk was > 7.5 $\log_{10}$ CFU/g at the end of 24 h incubation and this was maintained for 14 d storage at 4ºC. After 24 h fermentation, there was a significant increase ($P < 0.05$) in soluble calcium possibly due to a pH decrease during fermentation. *L. acidophilus* ATCC4962 and *L. plantarum* ASCC 276 demonstrated high increase in calcium solubility.

Selected *Lactobacillus* spp. produced phytase in varying degrees depending on the strain. Phytase activity was observed when the strains were added to a modified MRS broth and in CFSM. *L. acidophilus* ATCC4161 showed the highest activity at 14 d storage at 5°C. The optimum pH for phytase activity was found to be around 5. The hydrolysis of phytic acid to its lower IPs during fermentation could not be observed in our study.

Significant level of bioconversion ($P < 0.05$) of isoflavones into biologically active aglycones by ß-glucosidase activity was observed during fermentation of CFSM with the *Lactobacillus* spp. *L. fermentum* VRI-003 had the highest levels of daidzein, glycetein and genistein at 24 h fermentation and all the other strains also had similar bioconversion levels. In summary, fermentation of CFSM with probiotics can potentially enhance calcium bioavailability due to increased calcium solubility,

bioactive isoflavone aglycone enrichment and possible phytic acid degradation due to the presence of phytase.

Labelling of the fortificant after soymilk manufacture followed by heat treatment (90°C for 30 mins) showed similar calcium distribution as when the fortificant was labelled prior to soymilk manufacture. Being the simplest and least time consuming method, the labelling of the fortificant after soymilk manufacture with heat treatment was used to label CFSM used in the *in vivo* studies. Binding of ^{45}Ca with the identified soy protein (α'β-conglycinin, α-β-conglycinin and β portion-glycinin) was not observed during the electrophoresis of labelled CFSM.

The *in vivo* study showed that the fractional calcium absorption from CFSM and from cows' milk in 12 osteopenic post menopausal women using a randomised single-blind crossover study was similar ($P > 0.05$). The mean fractional calcium absorption (α) values were 0.65 (± 0.19) and 0.66 (± 0.22) respectively. This CFSM can therefore be used as a substitute for cows' milk as a rich calcium source in the diets of post menopausal women, vegetarians and individuals with lactose intolerance or milk allergies. Only one study participant was a confirmed equol producer. This study could not confirm if equol production status contributes to increased calcium absorption from soymilk due to the sample size limitation.

The mean fractional calcium absorption values from the consumption of non fermented CFSM and fermented CFSM with *Lactobacillus acidophilus* ATCC4962 were 0.65 (± 0.19) and 0.71 (± 0.29) respectively. Fermentation of CFSM did not significantly ($P = 0.122$) enhance calcium absorption in osteopenic post menopausal women.

The research investigated a comparative method of measuring calcium absorption from various types of milk or calcium fortified milk. This method may be adapted for similar comparative studies. The research has shown that calcium absorption from calcium fortified soymilk in this case was comparable to cows' milk. Investigation of ways to increase calcium bioavailability was investigated via the fermentation of calcium fortified soymilk using probiotics. The *in vitro* studies were done to explore the potential benefits of fermentation of calcium fortified soymilk.

According to the *in vitro* studies, the fermentation of CFSM with probiotics may be a promising way to enhance calcium bioavailability due to increasing of calcium solubility, phytase activity, and aglycone bioconversion producing genistein, diadzein and glycetein. These isoflavones have shown to have a bone sparing effect. Genistein can increase bone formation, osteoblast number, and osteocalcin level which might be responsible for bone-sparing effects of genistein. The fermentation of calcium fortified soymilk also provides the known beneficial effects of probiotic consumption. Longer feed studies using the fermented fortified soymilk with larger sample size are required to determine beneficial effects *in vivo*.

10.2 Future directions for research

Further studies are required to investigate the potential phytic acid hydrolysis by *Lactobacillus* spp. in other soymilks made from soybeans instead of soy protein isolate based soymilk. Soymilk made from soybeans would have higher amounts of natural phytate. Also, it is recommended that further studies are undertaken to analyse the calcium binding properties of the soy proteins from CFSM available commercially so

that clear fortification methods and formulas are achieved to ensure maximum calcium bioavailability.

Addition of fortificants can be an effective way to ensure an adequate intake of dietary calcium while fermentation of soymilk can provide cumulative long term health benefits however; longer studies using different types of commercially available soymilk; with a larger sample size study would be required to examine the effects of long term CFSM consumption (fermented and non fermented) on bone mineral density. The suggested research will confirm CFSM's (fermented and non fermented) potential in promoting calcium bioavailability and bone health. The mechanism of action in protecting bone health would also need to be determined. In parallel, the investigation if equol producers express higher fractional calcium absorption values that non equol producers from food sources, especially soy, would be beneficial in explaining the differences in calcium metabolism between individuals.

REFERENCES

Abrams, S. A. (1999). Using stable isotopes to assess mineral absorption and utilization by children. *American Journal of Clinical Nutrition, 70*(6), 955-964.

Abrams, S. A., Yergey, A. L., & Heaney, R. P. (1994). Relationship between balance and dual tracer isotopic measurements of calcium absorption and excretion. *Journal of Clinical Endocrinology and Metabolism, 79*(4), 965-969.

Adlercreutz, C. H., Goldin, B., Gorbach, S., Hockerstedt, K., Watanabe, S., Hamalainen, E., et al. (1995). Soybean phytoestrogen intake and cancer risk. *Journal of Nutrition, 125*(3 supplement), 757S-770S.

Alekel, D. L., Germain, A. S., Peterson, C. T., Hanson, K. B., Stewart, J. W., & Toda, T. (2000). Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. *American Journal Of Clinical Nutrition, 72*, 844-852.

Al-Walsh, I., Horner, H. T., Palmer, R. G., Reddy, M. B., & Massey, L. K. (2005). Oxalate and phytate of soy foods. *Journal of agricultural and food chemistry, 53*, 5670-5674.

Anderson, J. W., Smith, B. M., & Washnock, C. S. (1999). Cardiovascular and renal benefits of dry bean and soybean intake. *American Journal Clinical Nutrition, 70*(3), 464S-474S.

Angelis, M. D., Gallo, G., Corbo, M. R., McSweeney, P. L. M., Faccia, M., Giovine, M., et al. (2003). Phytase activity in sour dough lactic acid bacteria: purification and characterization of a phytase from Lactobacillus sanfranciscensis CB1. *International Journal of Food Microbiology, 87*(2003), 259-270.

Arjmandi, B., Birnbaum, R., & Goyal, N. V. (1998). Bone-sparing effect of soy protein in ovarian hormone-deficient rats is related to its isoflavone content. *American Journal Clinical Nutrition, 68*, 1364S-1368S.

Arjmandi, B. H., Alekel, L., & Hollis, B. W. (1996). Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. *Journal of Nutrition, 126*, 161-167.

Ashton, J. F. (2009). Berkeley Vale New South Wales 2261: Sanitarium Health Food Company.

Atkinson, C., Compston, J. E., Day, N. E., Dowsett, M., & Bingham, S. A. (2004). The effects of phytoestrogen isoflavones on bone density in women: a double blind, randomized, placebo-controlled trial. *American Journal Of Clinical Nutrition, 79*, 326-333.

Atmaca, A., Kleerekoper, M., Bayraktar, M., & Kucuk, O. (2008). Soy Isoflavones in the Management of Postmenopausal Osteoporosis: Mechanism of Action of Soy Isoflavones. *Menopause, 15*(4), 748-757.

Awumey, E. M., & Bukoski, R. D. (2006). Cellular functions and fluxes of calcium. In C. M. Weaver & R. P. Heaney (Eds.), *Calcium in Human Health*. Totowa, NJ: Humana Press Inc.

Axelson, M., Kirk, D. N., Farrant, R. D., Cooley, G., Lawson, A. M., & Setchell, K. D. R. (1982). The identification of the weak oestrogen equol [7-hydroxy-3-(4'-hydroxyphenyl)-chroman] in human urine. *Journal of Biochemistry, 201*, 353-357.

Axelsson, L. (2004). Lactic Acid Bacteria: Classification and Physiology. In A. Von Wright (Ed.), *Lactic Acid Bacteria* (pp. 1-66). New York, USA: Marcel Dekker Inc.

Bae, H. D., Yanke, L. J., Cheng, K., & Selinger, L. (1999). A novel staining method for detecting phytase activity. *Journal of microbiological methods, 39*, 17-22.

Bao, X. L., Song, M., Zhang, J., Chen, Y., & Guo, S. T. (2007). Calcium-binding ability of soy protein hydrolysates. *Chinese Chemical Letters, 18*(9), 1115-1118.

Bhandarkar, S. D., Bluhm, M. M., MacGregor, J., & Nordin, B. E. C. (1961). An isotope test of calcium absorption. *British Medical Journal, 2*, 1539-1541.

Bohn, T., Davidsson, L., Walczyk, T., & Hurell, R. F. (2004). Phytic acid added to white-wheat bread inhibits fractional apparent magnesium absorption in humans. *American Journal of Clinical Nutrition, 79*, 418-423.

Bradford, M. M. (1976). A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248-254.

Bronner, F. (1998). Calcium absorption - a paradigm for mineral absorption. *Journal of Nutrition, 128*, 917-920.

Bronner, F. (2009). Recent developments in intestinal calcium absorption. *Nutrition Review, 67*, 109-113.

Bronner, F., & Abrams, S. A. (1998). Development and regulation of calcium metabolism in Healthy girls. *The Journal of Nutrition, 128*(9), 1474-1480.

Brouns, F. (2002). Soya isoflavones: a new and promising ingredient for the health foods sector. *Food Research International, 35*, 187-193.

Cai, D. J., Zhao, Y. D., Glasier, J., Cullen, D., Barnes, S., Turner, C. H., et al. (2005). Comparative effect of soy protein, soy isoflavones, and 17 beta-estradiol on bone metabolism in adult ovariectomized rats. *Journal of Bone and Mineral Research, 20*(5), 828-839.

Calcium, Vitamin D and Osteoporosis - A guide for consumers. (2008). Retrieved 14 October 2008, from http://www.osteoporosis.org.au/files/internal/CalciumVitD_consumer.pdf

Camacho, P. M., & Miller, P. D. (2007). *Osteoporosis: A guide for clinicians*. Philadelphia: Wolters Kluwer Lippincott Williams & Wilkins.

Castelo-Branco, C., Palacios, S., Calaf, J., Vázquez, F., & Lanchares, J. L. (2005). Available medical choices for the management of menopause. *Maturitas Supplement: Management of the Asymptomatic Postmenopusal Women. Proceedings from the Amsterdam Menopause Symposium, October 3, 2004, Amsterdam, The Netherlands, 52*(Supplement 1), 61-70.

Cederroth, C. R., & Nef, S. (2009). Soy, phytoestrogens and metabolism: A review. *Molecular and Cellular Endocrinology Special Issue: Endocrine Disruptors from the Environment in the Aetiology of Obesity and Diabetes, 304*(1-2), 30-42.

Charoenkiatkul, S., Kriengsinyos, W., Tuntipopipat, S., Suthutvoravut, U., & Weaver, C. M. (2008). Calcium absorption from commonly consumed vegetables in healthy Thai women. *Journal of Food Science, 73*(9), H 218 - 221.

Chen, Y. M., Ho, S. C., Lam, S. S. H., Ho, S. S. S., & Woo, J. L. F. (2003a). Soy Isoflavones have a favorable effect on bone loss in chinese postmenopausal women with lower bone mass: A double-blind, randomized, controlled trial. *Journal of Clinical Endocrinology & Metabolism, 88*(10), 4740-4747.

Chen, Y. M., Ho, S. C., Lam, S. S. H., Ho, S. S. S., & Woo, J. L. F. (2003b). Soy Isoflavones have a favorable effect on bone loss in chinese postmenopausal

women with lower bone mass: A double-blind, randomized, controlled trial. *The Journal of Clinical Endocrinology & Metabolism, 88*(10), 4740-4747.

Cherma, D., Coomarasamt, A., & El-Toukhy, T. (2007). No-hormaonal theraphy of post-menopausal vasomotor symtoms; a structured evidence-based review. *Archives of Gynecology Obstetrics, 276*, 463-469.

Chiechi, L. M., Putignano, G., Guerra, V., Schiavelli, M. P., Cisternino, A. M., & Carriero, C. (2003). The effect of a soy rich diet on the vaginal epithelium in postmenopause: a randomized double blind trial. *Maturitas, 45*(4), 241-246.

Chien, H.-L., Huang, H.-Y., & Chou, C.-C. (2006). Transformation of isoflavone phytoestrogen during the fermentation of soymilk with lactic acid bacteria and *Bifidobacteria*. *Food Microbiology, 23*, 772-778.

Choi, Y.-B., Kim, K.-S., & Rhee, J.-S. (2002). Hydrolysis of soybean isoflavone glucosides by lactic acid bacteria. *Biotechnology Letters, 24*, 2113-2116.

Chun, J., Kim, G. M., Lee, K. W., Cho, I. D., Kwon, G.-H., Park, J.-Y., et al. (2007). Conversion of Isoflavone Glucosides to Aglycones in Soymilk By Fermentation with Lactic Acid Bacteria. *Journal of Food Science, 72*(2), M39-M44.

Chung, J., Kim, G. M., Lee, K. W., Cho, I. D., Kwon, G.-H., Park, J.-Y., et al. (2007). Conversion of Isoflavone Glucosides to Aglycones in Soymilk By Fermentation with Lactic Acid Bacteria. *Journal of Food Science, 72*(2), M39-M44.

Commane, D., Hughes, R., Shortt, C., & Rowland, I. (2005). The potential mechanisms involved in the anti-carcinogenic actions of probiotics. *Mutation Research, 591*, 276-289.

Damodaran, S., & Paraf, A. (1997). *Food Protein And Their Applications*. New York: Marcel Dekker, Inc.

De Angelis, M., Gallo, G., Corbo, M. R., McSweeney, P. L. M., Faccia, M., Giovine, M., et al. (2003). Phytase activity in sour dough lactic acid bacteria: purification and characterization of a phytase from Lactobacillus sanfranciscensis CB1. *International Journal of Food Microbiology, 87*(2003), 259-270.

De Man, J. D., Rogosa, M., & Sharpe, M. E. (1960). A Medium for the Cultivation of Lactobacilli. *Journal of Applied Bacteriology, 23*, 130-135.

De Mann, J. D., Rogosa, M., & Sharpe, M. E. (1960). A Medium for the Cultivation of Lactobacilli. *Journal of Applied Bacteriology, 23*, 130-135.

de Sousa-Muñoz, R. L., & Filizola, R. G. (2009). Efficacy of soy isoflavones for depressive symptoms of the climacteric syndrome. *Maturitas, 63*(1), 89-93.

Dellaglio, F., & Giovanna, E. F. (2005). Taxonomy of Lactobacilli and Bifidobacteria. In G. W. Tannock (Ed.), *Probiotics and Prebiotics: Scientific Aspects*. Norfolk: Caister Academic Press.

Dog, T. L. (2005). Menopause: a review of botanical dietary supplements. *American Journal of Medicine, 118*(12B), 985-1085.

Donkor, O. N., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2006). Effect of acidification on the activity of probiotics in yoghurt during cold storage. *International Dairy Journal, 16*, 1181-1189.

Egli, I., Davidsson, L., Zeder, C., Walczyk, T., & Hurell, R. (2004). Dephytinization of a complementary food based in wheat and soy increases zinc, but not copper, apparent absorption in adults. *Journal of Nutrition, 134*, 1077-1080.

Fairweather - Tait, S., Fox, T. E., Harvey, L. J., Teucher, B., & Dainty, J. (2001). Methods for analysis of trace-element absorption. In M. Jackson (Ed.), *Advances in Isotope Methods for the Analysis of Trace Elements in Man*: CRC Press.

Fanti, P., Monier-Faugere, M. C., & Geng, Z. (1998). The phytoestrogen genistein reduces bone loss in short-term ovariectomized rats. *Osteoporosis International, 8*, 274-281.

Fisher, S. K., Novak, J. E., & Agranoff, B. W. (2002). Inositol and higher inositol phosphates in neural tissues: homeostasis, metabolism, and functional significance. *Journal of Neurochemistry, 82*, 736-754.

Fiske, C. H., & Subbarow, Y. (1925). The Colorimetric Determination of Phosphorus. *Journal of Biological Chemistry, 66,*, 375-400.

Fonseca, D., & Ward, W. E. (2004). Daidzein together with high calcium preserve bone mass and biomechanical strength at multiple sites in ovariectomized mice. *Bone, 35*, 489-497.

Fontaine, E. A., Claydon, E., & Tayler-Robinson, D. (1996). *Lactobacilli* from women with or without bacterial vaginosis and observations on the significance of hydrogen peroxide. *Microbial Ecology in Health and Disease, 9*, 135-141.

Fuller, R. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology, 66*(365-378).

Fumalaro, G., DeSimone, C., Pandey, V., Sahu, A. R., & Minisola, G. (2005). Probiotic lactobacilli: an innovative tool to correct the malabsorption syndrome of vegeterians? *Medical Hypotheses, 65*, 1132-1135.

Ganzle, M. G., Zhang, C., Monang, B.-S., Lee, V., & Schwab, V. L. (2009). Novel metabolites from cereal-associated lactobacilli-Novel functionalities for cereal products? *Food Microbiology, 26*, 712-719.

Gardner, C. D., Chatterjee, L. M., & Franke, A. A. (2009). Effects of isoflavone supplements vs. soy foods on blood concentrations of genistein and daidzein in adults. *The Journal of Nutritional Biochemistry, 20*(3), 227-234.

Geller, S., & Studee, L. (2005). Botanical and dietary supplements for menopausal symtoms; What works , what does not. *Journal of Women's Health, 14*(7), 634-649.

Gibson, G. R., Rastall, R. A., & Fuller, R. (2003). The Health Benefits of Probiotics and Prebiotics. In R. Fuller & G. Perdigon (Eds.), *Gut Flora, Nutrition, Immunity and Health* (pp. 52-69): Blackwell Publishing Ltd.

Goktepe, I., Juneja, V. K., & Ahmedna, M. (2006). *Probiotics in Food Safety and Human Health*. Boca Raton, FL: CRC Press Taylor & Francis Group.

Golbitz, P., & Jordan, J. (2006). Soyfoods: Market and Products. In M. N. Riaz (Ed.), *Soy Applications in Food*. Boca Raton: CRC Press.

Gonnelli, S., Campagna, M. S., Montagnani, A., Caffarelli, A., Cadirni, A., Giorgi, G., et al. (2007). Calcium bioavailability from a new calcium-fortified orange beverage, compared with milk, in health volunteers. *International Journal For Vitamin and Nutrition Research, 77*(4), 249-254.

Gracia, M. C., Torre, M., Marina, M. L., & Laborda, F. (1997). Composition and characterization of soya bean and related products. *Critical Review of Food Science and Nutrition, 37*, 361-391.

Gueguen, L., & Pointillart, A. (2000). The bioavailability of dietary calcium. *Journal of the American College of Nutrition, 19*, 119S-136S.

Gueldner, S. H., Grabo, T. N., Newman, E. D., & Cooper, D. R. (2008). *Osteoporosis: Clinical guidelines for prevention, diagnosis and management*. New York: Springer Publising Company.

Hambridge, K. M., Krebs, N. F., Westcott, J. L., Sian, L., Miller, L. V., Peterson, K. L., et al. (2005). Absorption of calcium from tortilla meals prepared from low-phytate maize. *American Journal Of Clinical Nutrition, 82*, 84-87.

Haraldsson, A. K., Veide, J., Andlid, T., Alminger, M. L., & Sandberg, A.-S. (2005). Degradation of phytate by high-phytase *Saccharomyces cerevisiae* strains during simulated gastrointestinal digestion. *Journal of Agricultural and Food Chemistry, 53*, 5438-5444.

Haros, M., Bielecka, M., & Sanz, Y. (2005). Phytase activity as a novel metabolic feature in *Bifidobacterium*. *FEMS Microbiology Letters, 247*, 231-239.

Haros, M., Carlsson, N. G., Almgren, A., Larsson-Alminger, M., Sandberg, A.-S., & Andlid, T. (2009). Phytate degradation by human gut isolated *Bifidobacterium pseudocatenulatum ATCC27919* and its probiotics potential. *International Journal of Food Microbiology, 135*, 7-14.

Heaney, R. P. (1999). Absorbing Calcium. *Clinical Chemistry, 45*, 161-162.

Heaney, R. P. (2000). Dietary protein and phosphorus do not affect calcium absorption. *American Journal of Clinical Nutrition, 72*, 758-761.

Heaney, R. P. (2001). Factors Influencing the Measurement of Bioavailability, Taking Calcium as a Model. *American Society for Nutritional Sciences*(Supplement), s1344-s1348.

Heaney, R. P. (2003). Quantifying human calcium absorption using pharmacokinetic methods. *Journal of Nutrition, 133*, 1224-1226.

Heaney, R. P. (2005). Osteoporosis: Protein, minerals, vitamins and other micronutrients. In A. Bendich & R. J. Deckelbaum (Eds.), *Preventive Nutrition: The Comprehensive Guide For Health Professionals* (Third ed.). Totowa, NJ: Humana Press Inc.

Heaney, R. P., Dowell, M. S., Rafferty, K., & Bierman, J. (2000). Bioavailability of the calcium in fortified milk, with some observations on method. *American Journal of Clinical Nutrition, 71*(5), 1166.

Heaney, R. P., Dowell, M. S., & Wolf, R. L. (2002). Estimation of true calcium absorption in men. *Clinical Chemistry, 48*(5), 786-788.

Heaney, R. P., & Layman, D. K. (2008). Amount and type of protein influences bone health. *American Journal of Clinical Nutrition, 87*(supplement), 1567-1570.

Heaney, R. P., Rafferty, K., & Bierman, J. (2005). Not all calcium-fortified beverages are equal. *Nutrition Today, 40*(1), 39-44.

Heaney, R. P., Rafferty, K., Dowell, M. S., & Bierman, J. (2005). Calcium fortification systems differ in bioavailability. *Journal of the American Dietetic Association, 105*(5), 807-809.

Heaney, R. P., & Recker, R. R. (1994). Determinants of endogenous fecal calcium in healthy women. *Journal of Bone and Mineral Research, 9*, 1621-1627.

Heaney, R. P., Weaver, C. M., & Recker, R. R. (1988). Calcium absorbability from spinach. *American Journal Of Clinical Nutrition, 47*, 707-709.

Hewison, M. (2012). An update on vitamin D and human immunity. *Clinical Endocrinology, 76*(315–325).

Ho, S., Chan, S., So, K., Yi, Q., & Sham, A. (2009). The Effect of Habitual Dietary Soy Isoflavone Intake Bone Mineral Density Changes in Perimenopausal Chinese Women. *Osteoporosis International, 20*, 161-161.

Ho, S. C., Chan, S. G., Yi, Q., Wong, E., & Leung, P. C. (2001). Soy Intake and the maintenance of peak bone mass in Hong Kong Chinese women. *Journal of bone and mineral research, 16*(7), 1363 -1369.

Hoogenkamp, H. W. (2005). *Soy Protein and Formulated Meat Products* (1st ed.). London: CABI Publishing.

Howes, L. G., Howes, J. B., & Knight, D. C. (2006). Isoflavone therapy for menopausal flushes: A systematic review and meta-analysis. *Maturitas, 55*(3), 203-211.

Huang, H.-Y., Yang, H.-P., Yang, H.-T., Yang, T.-C., Shieh, M.-J., & Huang, S.-Y. (2006). One-year soy isoflavone supplementation prevents early postmenopausal bone loss but without a dose-dependent effect. *The Journal of Nutritional Biochemistry, 17*(8), 509-517.

Hughes, D. B., & Hoover, D. G. (1995). Viability and enzymic activity of bifidobacteria in milk. *Journal of Dairy Science, 78*, 268-276.

Huntley, A. L., & Ernst, E. (2004). Soy for the treatment of perimenopausal symptoms--a systematic review. *Maturitas, 47*(1), 1-9.

Hurell, R. F., Reddy, M. B., Huillerat, M.-A., & Cook, J. D. (2003). Degradation of phytic acid in cereal porridges improves iron absorption by human subjects. *American Journal Of Clinical Nutrition, 77*, 1213-1219.

Hutchins, A. M., Slavin, J. L., & Lampe, J. W. (1995). Urinary isoflavonoid phytoestrogen and lignan excretion after consumption of fermented and unfermented soy products. *Joournal of American Dietetics Association, 95*, 545-551.

Isiguro, T., Ono, T., Nakasato, K., & Tsukamoto, C. (2005). Rapid Measurement of phytate in soy products by mid-infrared spectroscopy. *Journal of Food Science, 70*(1), 63-66.

Isolauri, E., Salminen, S., & Ouwehand, A. C. (2004). Probiotics. *Best Practice & Research Clinical Gastroenterology, 18*(2), 299-313.

Izumi, T., Piskula, M. K., Osawa, S., Obata, A., Tobe, K., Saito, M., et al. (2000). Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *Journal of Nutrition, 130*, 1695-1699.

Jackman, R. H., & Black, C. A. (1951). Solubility of iron, aluminium, calcium and magnesium inositol phosphates at different pH values. *Soil Science, 72*, 179-186.

Jariwalla, R. J., Sabin, R., Lawson, S., & Herman, Z. S. (1990). Lowering of serum cholesterol and triglycerides and modulation of divalent cations by dietary phytase. *Journal of Applied Nutrition, 42*, 18-28.

Jefferson, A. (2005). Phytoestrogens and the menopause - do they really help? *Nutrition Bulletin, 30*, 370-373.

Kano, M., Takayanagi, T., Harada, K., Sawada, S., & Ishikawa, F. (2006). Bioavailability of isoflavones after ingestion of soy beverages in healthy adults. *Journal of Nutrition, 136*, 2291-2296.

Kerstetter, J. E., O'Brien, K. O., Caseria, D. M., Wall, D. E., & Insogna, K. L. (2005). The impact of dietary protein on calcium absorption and kinetic measures of bone turnover in women. *Journal of Clinical Endocrinology and Metabolism, 90*(1), 26-31.

Khatib, K. A., Herald, T. J., Aramouni, F. M., MacRitchie, F., & Schapaugh, W. T. (2002). Characterization and Functional properties of soy β-Conglycinin and Glycinin of Selected Genotypes. *Journal of Food Science, 67*(8), 2923-2929.

Kok, L., Kreijkamp-Kaspers, S., Grobbee, D. E., Lampe, J. W., & van der Schouw, Y. T. (2005). Soy isoflavones, body composition, and physical performance. *Maturitas, 52*(2), 102-110.

Kolida, S., Saulninier, D. M., & Gibson, G. R. (2006). Gastrointestinal Microflora: Probiotics. *Advances in Applied Microbiology, 59*(187-219).

Kruger, M. C., & Horrobin, D. F. (1997). Calcium metabolism, osteoporosis and essential fatty acids: A review. *Progress in Lipid Research, 36*(2-3), 131-151.

Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., Van Der Saag, P. T., et al. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogens receptor beta. *Endocrinology, 139*, 4252-4263.

Kumagai, H., Ishida, S., Koizumi, A., Skurai, H., & Kumangai, H. (2002). Preparation of phytate-removed deaminated soybean globulins by ion exchangers and characterization of their calcium binding ability. *Journal of Agricultural and Food Chemistry, 50*, 172-176.

Kumagai, H., Koizumi, A., Sato, N., Ishikawa, Y., Suda, A., Sakurai, H., et al. (2004). Effect of phytate-removal and deamination of soybean proteins on calcium absorption in the situ rats. *Biofactors, 22*, 21-24.

Laemmli, U. R. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature, 227*, 680-685.

Lee, Y.-B., Lee, H. J., & Sohn, H. S. (2005). Soy isoflavones and cognitive function. *The Journal of Nutritional Biochemistry, 16*(11), 641-649.

Lehrfeld, J. (1994). HPLC separation and quantitation of phytic acid and some inositol phosphates in foods: Problems and solutions. *Journal of Agricultural Food Chemistry, 42*, 2726-2731.

Levis, S., Strickman-Stein, N., Doerge, D. R., & Krischer, J. Design and baseline characteristics of the Soy Phytoestrogens As Replacement Estrogen (SPARE) study -- A clinical trial of the effects of soy isoflavones in menopausal women. *Contemporary Clinical Trials, In Press, Corrected Proof*.

Lilly, D. M., & Stillwell, R. H. (1965). Probiotics: growth promoting factors produced by other microorganisms. *Science, 147*, 747-748.

Lionetti, E., Indrio, F., Pavone, L., Borrelli, G., Cavallo, L., & Francavilla, R. (2010). Role of Probiotics in Pediatric Patients with *Helicobacter pylori*

Infection: A Comprehensive Review of the Literature. *Helicobacter, 15*(2), 79-87.

Liong, M. T., & Shah, N. P. (2005). Bile salt deconjugation and BSH activity of five bifidobacterial strains and their cholesterol co-precipitating properties. *Food Research International, 38*, 135-142.

Liu, K. (1997). *Soybeans: Chemistry, Technology and Utilization*. New York: Chapman & Hall.

Liu, K. (2004). *Soybeans as Functional Foods and Ingredients*. Champaign, Illinois: AOCS Press.

Lonnerdal, B. (2002). Phytic acid-trace element (Zn, Cu, Mn) interactions. *International Journal of Food Science and Technology, 37*, 749-758.

Lonnerdal, B., Jayawickrama, L., & Lien, L. E. (1999). Effect of reducing the phytate content and of partially hydrolyzing the protein in soy formula on zinc and copper absorption and status in infant rhesus monkeys and rat pups. *American Journal Of Clinical Nutrition, 69*, 490-496.

Lopez - Huertas, E., Teucher, B., Boza, J. J., Martinez - Ferez, A., Majsak - Newman, G., Baro, L., et al. (2006). Absorption of calcium from milks enriched with fruto-oligosaccharides, tricalcium phosphate, and milk solids. *American Journal Of Clinical Nutrition, 83*, 310-316.

Lopez, H. W., Leenhardt, F., Coudray, C., & Remesy, C. (2002). Minerals and phytic acid interactions: Is it a real problem for human nutrition? *International Journal of Food Science and Technology, 37*, 727-739.

Lowry, O. H., & Lopez, J. A. (1946). The Determination of Inorganic Phosphate in the Presence of Labile Phosphate Esters. *Journal Biological Chemistry, 162*, 421-428.

Lydeking-Olsen, E., Beck-Jensen, J. E., Setchell, K. D. R., & Holm-Jensen, T. (2004). Soymilk or progesterone for prevention of bone loss. *European Journal of Nutrition, 43*, 246-257.

Lydeking-Olsen, E., Jensen, J. B., Setchell, K. D. R., Damhus, M., & Jensen, T. H. (2002). Isoflavone-rich soymilk prevents bone-loss in the lumbar spine of postmenopausal women. A 2 year study. *Journal of Nutrition, 132*, 581.

Ma, S. J., Leng, B., Xu, X. Q., Zhu, X. Z., Shi, Y., Tao, Y. M., et al. (2011). Purification and characterization of beta-1,4-glucosidase from *Aspergillus glaucus*. *African Journal of Biotechnology, 10*(84), 19607-19614.

Management of osteoporosis in postmenopausal women: 2010 position statement of The North American Menopause Society. (2010). *Menopause, 17*, 25-54.

Marshall, D. H. (1976). Calcium and phosphate kinetics. In B. E. C. Nordin (Ed.), *Calcium, phosphate and magnesium metabolism* (pp. 257-297). Edinburgh: Churchill Livingstone.

Marshall, D. H., & Nordin, B. E. C. (1969). Kinetic Analysis of plasma radioactivity after oral ingestion of radiocalcium. *Nature, 22*, 797.

Marshall, D. H., & Nordin, B. E. C. (1981). A comparison of radioactive calcium absorption tests with net calcium absorption. *Clinical Science, 61*, 477-481.

Martin, M. E., Haourigui, M., Pelissero, C., Benassayag, C., & Nunez, E. A. (1996). Interactions between phytoestrogens and human sex steroid binding protein. *Life Science, 58*, 429-436.

Mathey, J., Mardon, J., Fokialakis, N., Puel, C., Kati-Coulibaly, S., Mitakou, S., et al. (2007). Modulation of soy isoflavones bioavailability and subsequent effects on bone health in ovariectomised rats: the case for equol. *Osteoporosis International, 18*, 671-679.

Matkovic, V., Fontana, D., Tominac, C., Goel, P., & Chestnut, C. H. (1990). Factors that influence peak bone mass formation: a study of calcium balance and the inheritence of bone mass in adolescent females. *Journal of Clinical Nutrition, 52*, 878-888.

Matsuura, M., & Obata, A. (1993). Glucosidases from soybeans hydrolyze daidzin and genistin. *Journal of Food Science, 58*, 144-147.

Matsuura, M., Obata, A., & Fukushima, D. (1989). Objectionable flavor of soy milk developed during the soaking of soybeans and its control. *Journal of Food Science, 54*, 602-605.

McCarron, D. A., & Heaney, R. P. (2004). Estimated healthcare savings associated with adequate dairy food intake. *American Journal of Hypertension, 17*, 88-97.

Messina, M., & Hughes, C. (2003). Efficacy of soyfoods and soybean isoflavone supplements for alleviating menopausal symptoms is positively related to initial hot flush frequency. *Journal Medicinal Food, 6*, 1-11.

Messina, M. J. (1999). Legumes and soybeans:overview of their nutritional profiles and health effects. *American Journal Of Clinical Nutrition, 70*(Suppl), 439S-450S.

Metchnikoff, E. (1907). *The Prolongation of Life: Optimistic Studies*. London, UK: William Heinemann.

Million Women Study Collaborators. (2003). Breast cancer and hormone replacement therapy in the Million Women Study. *Lancet, 362*, 419-427.

Million Women Study Collaborators. (2007). Ovarian cancer and hormone replacement therapy in the Million Women Study. *Lancet, 369*, 1703-1710.

Miquel, J., Ramírez-Boscá, A., Ramírez-Bosca, J. V., & Alperi, J. D. (2006). Menopause: A review on the role of oxygen stress and favorable effects of dietary antioxidants. *Archives of Gerontology and Geriatrics, 42*(3), 289-306.

Morito, K., Hirose, T., Kinjo, J., HIrakawa, T., Okawa, M., Nohara, T., et al. (2001). Interaction of phytoestrogens with estrogen receptors α and β. *Biological & Pharmaceutical Bulletin, 24*, 351-356.

Moser-Veillon, P. B., Mangels, A. R., Vieira, N. E., Yergey, A. L., Patterson, K. Y., Hill, A. D., et al. (2001). calcium fractional absorption and metabolism assessed using stable isotopes differ between postpartum and never pregnant women. *Journal of Nutrition, 131*, 2295-2299.

Murkies, A. L., Wilcox, G., & Davis, S. R. (1998). Phytoestrogens. *Journal of Clinical Endocrinology and Metabolism, 83*(2), 297-303.

Nelson, H. D. (2008). Menopause. *The Lancet, 371*(9614), 760-770.

Nickel, K. P., Berdine, M. R., Smith, D. L., Smith, J. B., Miller, G. D., & Weaver, C. M. (1996). Calcium bioavailability from bovine milk and dairy products in premenopausal women using intrinsic and extrinsic labeling techniques. *Journal of Nutrition, 126*, 1406-1411.

Nik, A. M., Tosh, S. M., Woodrow, L., Poysa, V., & Corredig, M. (2009). Effect of soy protein subunit composition and processing conditions on stability and particle size distribution of soymilk. *Lwt-Food Science and Technology, 42*(7), 1245-1252.

Nordin, B. E. C. (1976). *Calcium, phosphate and magnesium metabolism: Clinical physiology and diagnostic procedures*. London: Churchill Livingstone.

Nordin, B. E. C. (1997). *Calcium in health and disease*. Retrieved 24 February 2009, from http://www.fao.org/docrep/W7336T/W7336T04.HTM

Nordin, B. E. C. (2008). Redefining Ostoeporosis. *Calcified Tissue International, 83*, 365-367.

Nordin, B. E. C., Morris, H. A., Horowitz, M., Coates, P. S., O'Loughlin, P. D., & Need, A. G. (2009). Calcium malabsorption does not cause secondary hyperparathyroidism. *Calcified Tissue International, 85*, 31-36.

Nordin, B. E. C., Morris, H. A., Wishart, J. M., Scopacasa, F., Horowitz, M., Need, A. G., et al. (1998). Modification and validation of a single-isotope radiocalcium absorption test. *Journal of Nuclear Medicine, 39*(1), 108-113.

Nordin, B. E. C., Need, A. G., & Morris, H. A. (1993). *Metabolic bone and Stone Disease* (Third ed.). New York: Churchill Livingston.

Nordin, B. E. C., Need, A. G., Morris, H. A., O'Loughlin, P. D., & Horowitz, M. (2004). Effect of age on calcium absorption in postmenopausal women. *American Journal Of Clinical Nutrition, 80*(4), 998-100.

Noureddini, H., & Dang, J. (2009). Degradation of phytates in distiller's grains and corn gluten feed by *Aspergillus niger* phytase. *Applied biochemistry and biotechnology, 159*, 11-23.

Okabe, Y., & Tanimoto, H. (2008). Effects of dietary intake of isoflavone aglygone-rich fermented soybean on bone metabolism in ovariectomized rats. *Journal of Health Science, 54*(3), 315-323.

Ong, L. (2007). *Influence of probiotic organisms on proteolytic pattern, release of bioactive compounds and sensory attributes of cheddar cheese.* Victoria University, Werribee, Vic.

Ong, L., Henriksson, A., & Shah, N. P. (2006). Development of probiotic cheddar cheese containing *Lactobacillus acidophilus, Lb. casei, Lb. paracasei* and *Bifidobacterium spp.* and the influence of these bacteria on proteolytic patterns and production of organic acid. *International Dairy Journal, 16*, 446-456.

Ong, L., & Shah, N. P. (2008). Influence of probiotic Lactobacillus acidophilus and L. Helveticus on proteolysis, Organic acid profiles, and ACE-Inhibitory activity of cheddar cheeses ripened at 4, 8 and 12^{o}C. *Journal Of Food Science, 73*(3), 111-120.

Ono, T., Katho, S., & Mothizuki, K. (1993). Influences of Calcium and pH on protein solubility in soybean milk. *Bioscience, Biotechnology and Biochemistry, 57*(1), 24-28.

Ososki, A. L., & Kennelly, E. J. (2003). Phytoestrogens: a review of the present state of research. *Phytotheraphy Research, 17*, 845-869.

Otieno, D. O., Ashton, J. F., & Shah, N. P. (2005). Stability of β−glucosidase activity produced by *Bifidobacterium* and *Lactobacillus spp.* in fermented soymilk during processing and storage. *Journal of Food Science, 70*(4), 236-241.

Otieno, D. O., Ashton, J. F., & Shah, N. P. (2006a). Evaluation of enzymic potential for biotransformation of isoflavone phytoestrogen in soymilk by *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus casei*. *Food Research International, 39*, 394-407.

Otieno, D. O., Ashton, J. F., & Shah, N. P. (2006b). Stability of isoflavone phytoestrogens in fermented soymilk with *Bifidobacterium animalis* Bb12 during storage at different temperatures. *International Journal of Food Science and Technology, 41*, 1-10.

Otieno, D. O., Ashton, J. F., & Shah, N. P. (2006c). Stability of isoflavone phytoestrogens in fermented soymilk with *Bifidobacterium animalis* Bb12 during storage at different temperatures. *International Journal of Food Science and Technology, 41*, 1182-1191.

Otieno, D. O., Ashton, J. F., & Shah, N. P. (2007). Role of microbial strain and storage temperatures in the degredation of isoflavone phytoestrogen in fermented soymilk with selected B-glucosidase producing *Lactobacillus casei* strains. *Food Research International, 40*, 371-380.

Ouwehand, A. C., & Vesterlund, S. (2004). Antimicrobial components from lactic acid bacteria. In S. Salmien, A. V. Wright & A. Ouwehand (Eds.), *Lactic Acid Bacteria Microbiological and Functional Aspects* (pp. 375-389). New York, US: Marcel Dekker Inc.

Palacios, M. C., Haros, M., Rosell, C. M., & Sanz, Y. (2005). Characterization of an acid phosphatase from lactobacillus penstosus: regulation and biochemical properties. *Journal of Applied Microbiology, 98*, 229-237.

Parker, R. B. (1974). Probiotics, the other half of the antibiotic story. *Animal Nutrition and Health, 29*, 4-8.

Patterson, K. Y., & Veillon, C. (2001). Stable Isotopes of Minerals as Metabolic Tracers in Human Nutrition Research. *Experimental Biology and Medicine, 226*, 271-282.

Petri Nahas, E., Nahás Neto, J., De Luca, L., Traiman, P., Pontes, A., & Dalben, I. (2004). Benefits of soy germ isoflavones in postmenopausal women with contraindication for conventional hormone replacement therapy. *Maturitas, 48*(4), 372-380.

Phillippy, B. Q. (2001). Stability of Plant and Microbial Phytases. In N. R. Reddy (Ed.), *Food Phytates* (pp. 107-128): CRC Press.

A picture of osteoporosis. (2008). Canberra: Australian Institute of Health and Welfare.

Potter, S. M., Baum, J. A., Teng, H., Stillman, R. J., & Erdman, J. W. J. (1998). Soy protein and isoflavones: their effect on blood lipids and bone density in postmenopausal women. *American Journal Of Clinical Nutrition, 70*(3), 439-450.

Poutanen, K., Flander, L., & Katina, K. (2009). Sourdough and cereal fermentation in a nutritional perspective. *Food Microbiology, 26*, 693-699.

Powers, J. G., & Gilchrest, B. A. (2012). What you and your patients need to know about vitamin D. *Seminars in cutaneous medicine and surgery, 31*(1), 2-10.

Raghavendra, P., & Halami, P. M. (2009). Screening selection and characterization of phytic acid degrading lactic acid bacteria from chicken intestine. *International Journal of Food Microbiology, 133*, 129-134.

Reale, A., Mannina, L., Tremonte, P., Sobolev, A., Succi, M., Sorrentino, E., et al. (2004). Phytate degradation by lactic acid bacteria and yeasts during the wholemeal dough fermentation: a 31P NMR study. *Journal of Agricultural and Food Chemistry, 52*, 6300-6305.

Reddy, N. R. (2001). *Food Phytates*. Hoboken: CRC Press.

Reid, G. (2006). Safe and efficacious probiotics: what are they? *Trends in Microbiology, 14*(8), 348-352.

Riaz, M. N. (2006). *Soy Applications in Food*. Boca Raton: CRC Press Taylor & Francis.

Rickard, S. E., & Thompson, L. U. (1997). Interactions and biological effects of phytic acid. In F. Shahidi (Ed.), *Antinutrients and Phytochemicals in Food* (pp. 294-312). Washington DC: ACS Symposiom Series 662, American Chemical Society.

Rickert, D. A., Johnson, L. A., & Murphy, P. A. (2004). Functional properties of improved glycinin and β-conglycinin fractions. *Journal of Food Science, 69*(4), 303-311.

Rimbach, G., Pallauf, J., Moehring, J., Kraemer, K., & Minihane, A. M. (2008). Effect of dietary phytate and microbial phytase on mineral and trace element bioavailability - A literature review. *Current topics in nutraceutcal research, 6*(3), 131-144.

Ross, R. P., Fitzgerald, G., Collins, K., & Stanton, C. (2002). Cheese delivering bioculturesprobiotic cheese. *The Australian Journal of Dairy Technology, 57*, 71-78.

Rossouw, J. E., Anderson, G. I., & Prentice, R. I. (2002). Risks and benefits of estrogen plus progestin to healthy postmenopausal women: principle results from the Women's Health Initiative randomized controlled trial. *Journal of American Medical Association, 288*(3), 321-333.

Rufer, C. E., Bub, A., Moseneder, J., Winterhalter, P., Sturtz, M., & Kulling, S. E. (2008). Pharmacokinetics of the soybean isoflavone diadzein in its aglycone and glucoside form: a randomised, doubleblind, crossover study. *The American Journal of Clinical Nutrition, 87*, 1314-1323.

Rüfer, C. E., Glatt, H., & Kulling, S. E. (2006). Structural elucidation of hydroxylated metabolites of the isoflavan equol by gas chromatography-mass spectrometry and high-performance liquid chromatography-mass spectrometry. *34*(1), 51-60.

Ryan-Borchers, T. A., Soon Park, J., Chew, B. P., McGuire, M., Fournier, L. R., & Beerman, K. A. (2006a). Soy isoflavones modulate immune function in healthy postmenopausal women. *The American Journal of Clinical Nutrition, 83*(5), 1118-1125.

Ryan-Borchers, T. A., Soon Park, J., Chew, B. P., McGuire, M., Fournier, L. R., & Beerman, K. A. (2006b). Soy isoflavones modulate immune function in healthy postmenopausal women. *American Journal of Clinical Nutrition, 83*(5), 1118-1125.

Sadler, M. J. (2004). Soy and health 2004: clinical evidence, dietetic applications. *Nutrition Bulletin, 30*, 385-391.

Sandberg, A.-S., & Andlid, T. (2002). Phytogenic and microbial phytase in human nutrition. *International journal of food science and technology, 37*, 823-833.

Sathyamoorthy, N., Wang, T. T. Y., & Phang, J. M. (1994). Stimulation of pS2 expression by diet-derived compounds. *Cancer Research, 54*, 957–961.

Schmid, K., Schlothauer, R.-C., Friedrich, U., Staudt, C., Apajalahti, J., & Hansen, E. B. (2006). Development of Probiotic Food Ingredients. In I. Goktepe, V. K. Juneja & M. Ahmedna (Eds.), *Probiotics in Food Safety and Human Health*. Boca Raton, FL: Taylor & Francis Group.

Schroder, B. G., Griffin, I. J., Specker, B. L., & Adrams, S. A. (2005). Absorption of calcium from the carbonated dairy soft drink is greater than that from fat-free milk and calcium-fortified orange juice in women. *Nutrition Research, 25*, 737-742.

Setchell, K. D. R. (1998). Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *American Journal Of Clinical Nutrition, 68(suppl)*, 1333S-1346S.

Setchell, K. D. R., Borriello, S. P., Hulme, P., Kirk, D. N., & Axelson, M. (1984). Nonsteroidal estrogens of dietary origin: possible roles in hormone dependent disease. *American Journal of Clinical Nutrition, 40*, 569-578.

Setchell, K. D. R., Brown, N. M., Desai, P., Zimmer-Nechemias, L., Wolfe, B. E., Brashear, W. T., et al. (2001a). Bioavailability of pure isoflavones in healthy humans and analysis od commercial soy isoflavone supplements. *Journal of Nutrition, 131*, 1362S-1375S.

Setchell, K. D. R., Brown, N. M., Desai, P., Zimmer-Nechemias, L., Wolfe, B. E., Brashear, W. T., et al. (2001b). Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *Journal of Nutrition, 131*, 1362S-1375S.

Setchell, K. D. R., Brown, N. M., & Lydeking-Olsen, E. (2002). The clinical importance of the metabolite equol - a clue to the effectiveness of soy and its isoflavones. *Journal of Nutrition, 132*, 3577-3584.

Setchell, K. D. R., & Cassidy, A. (1999a). Dietary isoflavones: Biological effects and relevance to human health. *The Journal of Nutrition, 129*(Supplement), 758s-767s.

Setchell, K. D. R., & Cassidy, A. (1999b). Dietary isoflavones: Biological effects and relevance to human health. *Journal of Nutrition, 129*(Supplement), 758s-767s.

Setchell, K. D. R., Clerici, C., Lephart, E. D., Cole, S. J., Heenan, C., Castellani, D., et al. (2005). S-Equol, a potent ligand for estrogen receptor β, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora. *American Journal of Clinical Nutrition, 81*, 1072-1079.

Setchell, K. D. R., & Cole, S. J. (2003). Variations in isoflavone levels in soy foods and soy protein isolates and issues related to isoflavone databases and food labeling. *Journal of Agricultural Food Chemistry, 51*, 4146-4155.

Setchell, K. D. R., & Cole, S. J. (2006). Method of Defining Equol-Producer Status and Its Frequency among Vegeterians. *Journal of Nutrition, 136*, 2188-2193.

Setchell, K. D. R., Faughnan, M. S., Avades, T., Zimmer-Nechemias, L., Brown, N. M., Wolf, B., et al. (2003). Comparing the pharmokinetics of diadzein and genistein with the use of ^{13}C-labeled tracers in premenopausal women. *American Journal of Clinical Nutrition, 77*, 411-409.

Setchell, K. D. R., & Lydeking-Olsen, E. (2003a). Dietary phytoestrogen and their effect on bone: evidence from in vitro and in vivo observational, and dietary

intervention studies. *American Journal Of Clinical Nutrition, 78(suppl)*, 593S-609S.

Setchell, K. D. R., & Lydeking-Olsen, E. (2003b). Dietary phytoestrogen and their effect on bone: evidence fron in vitroand in vivo observational, and dietary intervention studies. *American Journal Of Clinical Nutrition, 78(suppl)*, 593S-609S.

Setchell, K. D. R., Maynard Brown, N., Desai, P. B., Zimmer-Nechemias, L., Wolfe, B., Jakate, A. S., et al. (2003). Bioavailability, disposition, and dose-response effects of soy isoflavones when consumed by healthy women at physiologically typical dietary intakes. *Journal of Nutrition, 133*, 1027-1035.

Shah, N. P. (2000). Probiotic Bacteria: Selective Enumaration and Survival in Dairy Foods. *Journal of Dairy Science, 83*, 894-907.

Shah, N. P. (2000a). Some beneficial effecs of probiotic bacteria. *Bioscience and Microflora, 19*, 99-106.

Shah, N. P., & Wu, C. (1999). Aflatoxin B1 binding abilities of probiotic bacteria. *Bioscience and Microflora, 18*, 43-48.

Shamsuddin, A. M. (1995). Inositol phosphate have novel anticancer function. *Journal of Nutrition, 125*, 725-732.

Shamsuddin, A. M. (2002). Anticancer function of phytic acid. *Journal of Food Science and Technology, 37*, 769-782.

Shapiro, R., & Heaney, R. P. (2003). Co-dependence of calcium and phosphorus for growth and bone development under varying deficiency. *Bone, 32*, 532-540.

Shen, X., Weaver, C. M., Kempa-Steczko, A., Martin, B. R., Phillippy, B. Q., & Heaney, R. P. (1998). An inositol phosphate as a calcium absorption enhancer in rats. *Journal of Nutritional Biochemistry, 9*(5), 298-301.

Shin, H.-S., Lee, J.-H., Pestka, J. J., & Ustunol, Z. (2000). Growth and viability of commercial *Bifidobacterium* spp in skim milk containing oligosaccharides and inulin. *Journal of Food Science, 65*(5), 884-887.

Shutt, D., & Braden, A. (1968). The significance of equol in relation to the oestrogenic responses in sheep ingesting clover with a high formononetin content. *Australian Journal of Agricultural Research, 19*(545-553).

Singh, G., Arora, S., Sharma, G. S., Sindhu, J. S., Kansal, V. K., & Sangwan, R. B. (2007). Heat stability and calcium bioavailability of calcium-fortified milk. *LWT, 40*, 625-631.

Skoglund, E., & Sandberg, A.-S. (2001). Methods for Analysis of Phytate. In N. R. Reddy (Ed.), *Food Phytates* (Vol. 127-137): CRC Press.

Song, Y., Paik, H. Y., & Juong, H. (2008). Soybean and soy isoflavone intake indicate a positive change in bone mineral density for 2 years in yound korean women. *Nutrition Research, 28*, 25-30.

Spence, L. A., Lipscomb, E. R., Cadogan, J., Martin, B., Wastney, M. E., Peacock, M., et al. (2005). The effect of soy protein and soy isoflavones on calcium metabolism in postmenopausal women: a randomized crossover study. *American Journal of Clinical Nutrition, 81*(4), 916-922.

Spence, L. A., Lipscombe, E. R., Cadogan, J., Martin, B. R., Peacock, M., & Weaver, C. M. (2001). *Effects of soy isoflavones on calcium metabolism in postmenopausal women.* Paper presented at the 4th International Symposium on the Role of Soy in Preventing and Treating Chronic Diseases.

Stanton, C., Ross, R. P., Fitzgerald, G., & Van Sinderen, D. (2005). Fermented fundtional foods based on probiotics and their biogenic metabolites. *Current opinion in biotechnology, 16*, 198-203.

Steer, T. E., & Gibson, G. R. (2002). The microbiology of phytic acid metabolism by gut bacteria and relevance for bowel cancer. *International Journal of Food Science and Technology, 37*(7), 783-790.

Taku, K., Melby, M. K., Kurzer, M. S., Mizuno, S., Watanabe, S., & Ishimi, Y. Effects of soy isoflavone supplements on bone turnover markers in menopausal women: Systematic review and meta-analysis of randomized controlled trials. *Bone, In Press, Uncorrected Proof*.

Tamang, J. P., Tamang, B., Schillinger, U., Guigas, C., & Holzapfel, W. (2009). Functional properties of lactic acid bacteria from ethnic fermented vegetales of the Himalayas. *International Journal of Food Microbiology, 135*, 28-33.

Tang, A. L., Shah, N. P., Wilcox, G., Walker, K. Z., & Stojanovska, L. (2007). Fermentation of Calcium-Fortified Soymilk with Lactobacillus: Effects on Calcium Solubility, Isoflavone Conversion, and Production of Organic Acids. *Journal of Food Science, 72*(9), 431-436.

Tang, A. L., Walker, K. Z., Wilcox, G., Strauss, B. J., Ashton, J. F., & Stojanovska, L. (2010). Calcium absorption in Australian osteopenic post-menopausal women: an acute comparative study of fortified soymilk to cows' milk. *Asia Pacific Journal of Clinical Nutrition, 19*(2), 243-249.

Tannock, G. W. (2005). *Probiotics and prebiotics: Scientific aspects*. Norfolk, UK: Caister Academic Press.

Tham, D., Gardner, C., & Haskell, W. (1998). Potential health benefits of dietary phyto-oestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *Journal of Clinical Endocrinology and Metabolism, 83*, 2223-2235.

Theobald, H. E. (2005). Dietary calcium and health. *Nutrition Bulletin, 30*, 237-277.

Tripathi, A. K., & Misra, A. K. (2005). Soybean - a consumate functional food: A review. *Journal of food Science and Technology, 42*(2), 111-119.

Tsangalis, D., Ashton, J. F., Mcgill, A. E. J., & Shah, N. P. (2002). Enzymic transformation of isoflavone phytoestrogen in soymilk by β-Glucosidase producing bifidobacteria. *Journal of Food Science, 67*(8), 3104-3113.

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

Tsangalis, D., & Shah, N. P. (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.

Utian, W. H., Archer, D. F., & Bachmann, G. A. (2008). Estrogen and progestogen use in post-menopausal women: 2008 position statement of the North American Menopause Society. *Menopause, 15*(4 Pt1), 584-602.

Vatanparast, H., Bailey, D. A., Baxter-Jones, A. D. G., & Whiting, S. J. (2007). The effects of dietary proein on bone mineral mass in young adults may be modulated by adolescent calcium intake. *Journal of Nutrition, 137*, 2674-2679.

Wang, T. T. Y., Sathyamoorthy, N., & Phang, J. M. (1996). Molecular effects of genistein on estrogen receptor mediated pathways. *Carcinogenesis, 17*, 271–275.

Ward, W. E., Kim, S., Chan, D., & Fonseca, D. (2005). Serum equol, bone mineral density and biomechanical bone strength differ among four mouse strains. *Journal of Nutritional Biochemistry, 16*, 743-749.

Warren, M. P., Shortle, B., & Dominguez, J. E. (2002). Use of alternative therapies in menopause. *Best Practice & Research Clinical Obstetrics & Gynaecology, 16*(3), 411-448.

Watkins, B. A., Reinwald, S., Li, Y., & Seifert, M. F. (2005). Protective actions of soy isoflavones and n-3 PUFAs on bone mass in ovariectomized rats. *The Journal of Nutritional Biochemistry, 16*(8), 479-488.

Weaver, C. M., & Cheong, J. (2005). Soy isoflavones and bone health: the relationship is still unclear. *Journal of Nutrition, 135*, 1243-1247.

Weaver, C. M., & Heaney, R. P. (2006). *Calcium in Human Health*. Totowa: Humana Press.

Weaver, C. M., Heaney, R. P., Martin, B. R., & Fitzsimmons, M. L. (1992). Extrinsic vs intrinsic labeling of the calcium in whole-wheat flour. *American Journal Of Clinical Nutrition, 55*, 452-454.

Weaver, C. M., & Kannan, S. (2001). Phytate and Mineral Bioavailability. In N. R. Reddy (Ed.), *Food Phytates*: CRC Press.

Whyte, M. P. (1993). Hypocalcaemia. In B. E. C. Nordin, A. G. Need & H. A. Morris (Eds.), *Metabolic Bone ans Stone Disease* (pp. 147-162). New York: Churchill Livingstone.

Wijeratne, S. S. K., & Cuppett, S. (2007). Soy isoflavones protect the intestine fromlipid hydroperoxide mediated oxidative damage. *Journal of Agricultural Food Chemistry, 55*, 9811-9816.

Wilkinson, R. (1976). Absorption of calcium, phosphorus and magnesium. In B. E. C. Nordin (Ed.), *Calcium, phosphate and magnesium metabolism* (pp. 36-112). Edinburgh: Churchill Livingstone.

Wong, V. C., Lim, C. E., Luo, X., & Wong, W. S. (2009). Current alternative and complementary therapies used in menopause. *Gynecological Endocrinology, 25*(3), 166-174.

Wuttke, W., Jarry, H., & Seidlová-Wuttke, D. (2007). Isoflavones--Safe food additives or dangerous drugs? *Ageing Research Reviews, 6*(2), 150-188.

Xu, X., Wang, H. J., Murphy, P. A., Cook, L., & Hendrich, S. (1994). Diadzein is a more bioavailable soymilk isoflavone then genistein in adult women. *Journal of Nutrition, 124*, 825-832.

Zafar, T. A., Weaver, C. M., Jones, K., Moore, D. R., & Barnes, S. (2004). Inulin effects on bioavailability of soy isoflavones and their calcium absorption enhancing ability. *Journal of Agricultural and Food Chemistry, 52*, 2827-2831.

Zhang, Y., Wang, G. J., Song, T., Murphy, P. A., & Hendrich, S. (1999). Urinary disposition of the soybean isoflavones diadzein, genistein and glycetein differs among humans with moderate fecal isoflavone degradation activity. *Journal of Nutrition, 129*, 957-962.

Zhao, Y., Martin, B. R., & Weaver, C. M. (2005). Calcium Bioavailability of Calcium Carbonate Fortified Soymilk Is Equivalent to Cow's Milk in Young Women. *Journal of Nutrition, 135*, 2379-2382.

Zhou, J.-R., & Erdman, J. W. J. (2005). Soy Consumption and Cancer Prevention. In A. Bendich & R. J. Deckelbaum (Eds.), *Preventive Nutrition: The Comprehensive Guide for Health Professionals* (3rd ed., pp. 123-156). New Jersey: Humana Press.

Zhu, Y., Suits, M. D. L., Thompson, A. J., Chavan, S., Dinev, Z., Dumon, C., et al. (2010). Mechanistic insights into a Ca^{2+}-dependent family of α–mannosidases in a human gut symbiont. *Nature Chemical Biology, 6*, 125-132.

APPENDIX A:

PARTICIPANT INFORMATION FOR STUDY

(CFSM VS COWS' MILK)

PARTICIPANT INFORMATION FOR STUDY BELOW:

'A comparison of calcium absorption from fortified soy milk and cow's milk in postmenopausal women'

Introduction

Postmenopausal women are often advised to increase their intake of soymilk because it is a natural source of phytoestrogens that can aid in reducing menopausal symptoms. Soy milk however is not naturally rich in calcium. A high intake of calcium is important after menopause, to reduce the risk of fracture and osteoporosis. Calcium is often therefore added to soy milk. We do not know however, if this added calcium is well absorbed. If it is not, then women who choose soy milk rather than cow's milk may be at a greater risk of developing osteoporosis. It is therefore important for us to establish how well calcium is absorbed from soy milk.

We would like to invite you to be part of a study that will compare calcium absorption from soy milk and cow's milk in an attempt to address this question. This research will form one important component of a higher degree (Doctor of Philosophy) investigating the potential health benefits associated with increased calcium absorption in postmenopausal women. The human dietary intervention study that you are now invited to join will be conducted by a student researcher (Ph.D. candidate at Victoria University), Anne Lise Tang, under the close supervision of experienced academic researchers from the areas of medicine, physiology and food science. They include:

Dr Gisela Wilcox (*BMedSc(Hons), MBBS(Hons), FRACP, MAACB, FRCPA)*, Clinical Nutrition & Metabolism Unit, Monash Medical Centre

Assoc. Prof. Boyd Strauss *(MBBS, PhD, FRACP*), Director, Body Composition Laboratory, Monash Medical Centre

Dr Karen Walker (*BSc(Hons), PhD, MND, APD)*, Nutrition & Dietetics Unit, Monash University Department of Medicine, Monash Medical Centre

Assoc. Prof. Lily Stojanovska (*BSc, MSc, PhD*), School of Biomedical Sciences, Victoria University

Purpose of study

This study will provide critical information to indicate how well calcium is absorbed from soy milk compared to cow's milk. Results from this study will provide information to the soy industry on the efficacy of current methods for fortifying soy beverages with calcium. This research project will also be of benefit to the general public. The results of this study will help manufacturers to improve the calcium fortification of soy products to prevent bone disease in our aging population, particularly in postmenopausal women. Osteoporosis and fractures are a great burden to individuals and costly both personally and to the government. For oestrogen deficient postmenopausal women a product that provides a rich, bio-available non-dairy source of calcium would be highly valuable.

Are you suitable to participate in this study?

The study requires the recruitment of postmenopausal, osteopenic women aged between 50-65 years who:

- Are reasonably healthy.
- Have no strict dietary patterns (e.g. strict vegetarians),
- Rarely consume soy foods (no more than 1-3 serves per month)
- Are willing to consume both cow's milk and soy milk.

You are not suitable to take part in the study if you:

- have taken hormone therapy (HT) in the past 2-3 years
- have an allergy or intolerance to dairy or soy
- use prescription or non-prescription drugs such as, antibiotics or medications for conditions such as osteoporosis
- have a serious medical condition such as a bleeding disorder, heart, liver, gastrointestinal (e.g. coeliac disease), or kidney disease.
- have a cardiac pacemaker
- have a known psychiatric condition (phone discussion, followed by questionnaire on the day of study)

- have unusually high or low levels of calcium or vitamin D in your blood.
- regularly consume more than 2 standard alcoholic drinks per day

Project Outline and Requirements

If you agree to participate in the study, you will be asked some questions about your age, medical history and general lifestyle. You will then be asked to come into the Body Composition Laboratory at Monash Medical Centre for two morning appointments, spaced three weeks apart. Assistance with travel costs will be provided if required. Results of the overall study will be given to the participants and any published information will also be sent to the participants.

At each appointment, some basic medical tests will be done such as weight, height and body circumferences and BIA (Bioelectrical Impedance Analysis is a non invasive, painless test which measures resistance to an electrical current from which body water can be calculated). Then, you will be given a milk drink and blood samples will be taken. You will be asked not to take any vitamin or mineral supplements during the time that you are on the study.

For the week leading up to your first appointment, you will be required to follow your usual diet but to avoid all foods that are particularly high in calcium. .On the day before the first test drink, you will be asked not to eat or drink (except water) after dinner until you come into the laboratory the next morning.

On the day of your first test drink, you will arrive at the Body Composition Laboratory, a blood sample (equal to about one teaspoon or 5 mL) will be taken by a registered nurse/medical practitioner. You will then be asked to drink a small quantity (20 mL) of either soy milk or cow's milk. Both kinds of milk will contain a small amount of radioactive calcium. Radioisotope calcium is required in the dose because the amount of calcium absorbed from the test drink can then be easily detectable and measured using the radioisotope calcium. Then a small glass of water (200mL) will be given to wash out the left over milk in your mouth.

A small dose of radioactive Calcium – 45 (^{45}Ca) will be added to the fortified soy or cow's milk. This will expose you to little risk from radiation as discussed below. After the test drink, six small blood samples (5ml during each withdrawal) will be taken over 2 hours. A total of 35ml of blood sample will be drawn during the test. Measurement of these samples will enable us to determine how well calcium from the two drinks is absorbed into the body.

The blood samples will be taken using a plastic cannula, typically used in the hospital, which will be inserted in the forearm vein. Using the plastic cannula will avoid puncturing the skin various times, therefore minimising discomfort and pain during withdrawals. All blood samples that will be taken will be analysed for calcium immediately and they will be destroyed at the end of the study.

At the end of the 2 hours, you will be offered a lunch meal and you will be free to leave. Before you do so, your height, weight and percentage of body fat will be measured and your blood pressure and pulse measurement will be taken.

After three weeks, when you will resume your normal diet and lifestyle, you will come into the laboratory again for a second test drink where you will follow all the same procedures.

Possible Risks

The risk levels experienced at your appointments will be very minor, similar to those faced when undertaking a medical examination. It is possible that you will experience minor discomfort when blood samples are taken. You may feel the needle passing through the skin or experience minor bruising near the puncture site. There is extremely low risk of infection. Any problems will be treated by the nurse. If you are unhappy with your level of discomfort withdrawal from the study will be advised.

During the study you will be asked on two occasions to drink milk which contains a radioactive isotope known as Calcium-45. Calcium-45 is assumed to be uniformly

distributed in mineral bone and retained with a biological half life of approximately 165 days. This means that the radiation of the calcium-45 will be reduced by half in approximately 6 months.

The estimated effective dose from the exposures in this study is 0.7 milliSievert. This amount of radiation is about the same as 5 months of natural background radiation. All people on earth are exposed to background radiation. Background radiation comes from the sun, the earth, the air and all around us. The ill effects at very high doses of radiation have been well-documented, for example increased life-threatening cancer rates and sometimes death have been reported in populations exposed to nuclear explosions or in patients undergoing radiotherapy treatment.

At very low doses of radiation, similar to those being received from being a participant in this study, the risks are not completely known and have to be estimated using theoretical models based on the very high radiation dose data. The acknowledged theoretical model suggests that the risk of inducing cancer is about 3 in 100,000. This model is based on a conservative approach and the actual risk may be a lot smaller. Compared to other risks in everyday life this risk is considered minor. This theoretical risk is approximately the same as travelling 3000 km by car, or travelling 30,000 km by commercial aircraft.

Possible Benefits

You will be able to find out how well you absorb calcium from soy milk and cow's milk. From this information, you will be able to obtain advice on:

- How much soy milk/cow's milk you need to consume to meet the calcium RDI (Recommended Daily Intakes)?
- If your calcium absorption is low, advice on whether you need to get your Vitamin D level checked by your general practitioner and if you need to take extra calcium as well.

If you fit the above criteria, the above information will be of great value to minimise the risk of fracture in the future.

Confidentiality and Disclosure of Information

The information that you provide will remain confidential. It will be stored in a de-identified (non-personal and specific, that is, no names will be kept) form with the code known to only one researcher (Anne Lise Tang). The code to the information that would allow you to be identified will be destroyed at the completion of the study. The only people who will have access to the data will be the researchers involved in the study. The data will be stored in a locked filing cabinet in a locked room at the Body Composition Laboratory, MMC. De-identified data will be transferred to a password protected computer. Printed and electronic data from the study will be kept for 7 years after publication of study results. Identifying data will then be destroyed by shredding or electronic deletion. Outcomes/results of the study will be available to the participants and any furthers details about the study can be requested.

Participation is Voluntary

Participation in this research project is voluntary. If you do not wish to take part you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the study at any stage.

Treatment for injuries

In the event that you suffer an injury as a result of participating in this project, hospital care and treatment will be provided by the public health service at no extra cost to you.

Complaints

Should you have any complaints (Research project no. 05087A), you may contact Malar Thiagarajan, Executive Officer of the Southern Health Human Research Ethics Committee on (03) 95943025.

Ethical Guidelines

This project is being carried out according to the *National Statement on Ethical Conduct in Research Involving Human* (June 1999) produced by the National Health and Medical

Research Council of Australia. This project has been approved by the Southern Health Human Research Ethics Committee and Monash University Standing Committee on Ethics in Research involving Humans (SCERH).

Any queries about your participation in this project may be directed to the researchers

(Anne Lise Tang ☎ 0401430507; Dr. Gisela Wilcox ☎ 0413 870 315; A/Professor Boyd Strauss

APPENDIX B:

PARTICIPANT INFORMATION FOR STUDY

(CFSM VS FERMENTED CFSM)

PARTICIPANT INFORMATION FOR STUDY BELOW:

'A comparison of calcium absorption from fortified soymilk and fermented fortified soymilk in post menopausal women'

Introduction

A high intake of calcium is important after menopause, to reduce the risk of fracture and osteoporosis. Therefore, it is important to find ways in which we can improve the availability of calcium from food sources such as fortified soymilk.

Post menopausal women are often advised to increase their intake of fortified soymilk because it is a natural source of phytoestrogens that can aid in reducing menopausal symptoms. These are plant compounds that have an oestrogen-like effect beneficial to post menopausal women who have low oestrogen levels. Yet soymilk also contains compounds, such as phytates, that can reduce calcium absorption. These inhibitory compounds in soymilk can be removed and levels of available phytoestrogens/isoflavones can be increased if the milk is fermented with nutritionally beneficial probiotic bacteria.

These particular bacteria are commonly found in commercially available fermented dairy products like yoghurt and are known to be safe for human consumption. Various clinical studies have shown that consumption of fermented fortified soymilk could result in higher calcium absorption and improvement of bone health than during the consumption of non fermented fortified soymilk. Since these fermented soymilks may be particularly helpful for post menopausal women, it is important to determine how well the calcium contained can be absorbed compared to non fermented fortified soymilk.

We would like to invite you to be part of a study that will compare calcium absorption from non fermented fortified soymilk and to fermented fortified soymilk in an attempt to address this question. This research will form one important component of a higher degree (Doctor of Philosophy) investigating the potential health benefits associated with increased calcium absorption in post menopausal women. The human dietary intervention study that you are now invited to join will be conducted by a student researcher (Ph.D. candidate at

Victoria University), Anne Lise Tang, under the close supervision of experienced academic researchers from the areas of medicine, physiology and food science. They include:

- Dr Gisela Wilcox (*BMedSc(Hons), MBBS(Hons), FRACP, MAACB, FRCPA)*, Clinical Nutrition & Metabolism Unit, Monash Medical Centre
- Assoc. Prof. Boyd Strauss *(MBBS, PhD, FRACP*), Director, Body Composition Laboratory, Monash Medical Centre
- Dr Karen Walker (*BSc(Hons), PhD, MND, APD)*, Nutrition & Dietetics Unit, Monash University Department of Medicine, Monash Medical Centre
- Assoc. Prof. Lily Stojanovska (*BSc, MSc, PhD*), School of Biomedical Sciences, Victoria University

Purpose of study

This study will provide critical information to indicate how well calcium is absorbed from non fermented fortified soymilk compared to fermented fortified soymilk. Results from this study will provide information to the food research industry on effective ways to optimise calcium absorption from various food sources and may also contribute to towards minimising the incidence of fractures from osteoporosis, particularly in post menopausal women. Osteoporosis and fractures are a great burden to individuals and costly both personally and to the government. For oestrogen deficient post menopausal women a product that provides a rich, bio-available non-dairy source of calcium would be highly valuable.

Are you suitable to participate in this study?

The study requires the recruitment of postmenopausal, osteopenic women aged between 50-659 years who:

- Are reasonably healthy.
- Have no strict dietary patterns (e.g. strict vegetarians),
- Are willing to consume both non fermented soymilk and fermented soymilk.

You are not suitable to take part in the study if you:

- have taken hormone therapy (HT) in the past year
- have an allergy to soy
- use prescription or non-prescription drugs such as, antibiotics or medications for conditions such as osteoporosis
- have a serious medical condition such as a bleeding disorder, heart, liver, gastrointestinal (e.g. coeliac disease), or kidney disease.
- have a cardiac pacemaker
- have a known psychiatric condition (phone discussion, followed by questionnaire on the day of study)
- have unusually high or low levels of calcium or vitamin D in your blood.
- regularly consume more than 3 standard alcoholic drinks per day

Project Outline and Requirements

If you agree to participate in the study, you will be asked some questions about your age, medical history and general lifestyle. You will then be asked to come into the Body Composition Laboratory at Monash Medical Centre for two morning appointments(between 8.30am to 10.00am), spaced three weeks apart. Travel costs will be reimbursed, if necessary.

At each appointment, some basic medical tests will be done such as weight, height and body circumferences and BIA (Bioelectrical Impedance Analysis is a non invasive, painless test which measures resistance to an electrical current from which body water can be calculated). Then, you will be given a milk drink and blood samples will be taken. You will be asked not to take any vitamin or mineral supplements during the time that you are on the study.

For the week leading up to your first appointment, you will be required to follow your usual diet but to avoid all foods that are particularly high in calcium. .On the day before the first test drink, you will be asked not to eat or drink (except water) after dinner until you come into the laboratory the next morning at 8.30am.

On the day of your first test drink, you will arrive at the Body Composition Laboratory, a blood sample (equal to about two teaspoon or 10 mL) will be taken by a registered nurse/medical practitioner. You will then be asked to drink a small quantity (20 mL) of either fortified soymilk or fermented fortified soymilk. Both soymilk will contain a small amount of radioactive calcium. Radioisotope calcium is required in the dose because the amount of calcium absorbed from the test drink can then be easily detectable and measured using the radioisotope calcium. Then a small glass of water (200mL) will be given to wash out the left over milk in your mouth.

A small dose of radioactive Calcium – 45 (^{45}Ca) will be added to the fortified soy or cows' milk. This will expose you to little risk from radiation as discussed below. After the test drink, six small blood samples (5ml during each withdrawal) will be taken over 2 hours. A total of 40ml of blood sample will be drawn during the test. Measurement of these samples will enable us to determine how well calcium from the two drinks is absorbed into the body.

The blood samples will be taken using a plastic cannula, typically used in the hospital, which will be inserted in the forearm vein. The level of discomfort and invasiveness will be similar to that experienced during a normal blood test. Using the plastic cannula will avoid puncturing the skin various times, therefore minimising discomfort and pain during withdrawals. All blood samples that will be taken will be analysed for vitamin D level and calcium immediately and the blood samples will be destroyed at the end of the study.

At the end of the 2 hours, you will be offered a lunch meal voucher redeemable at the Monash Medical Centre café and you will be free to leave.

After three weeks, when you will resume your normal diet and lifestyle, you will come into the laboratory again for a second test drink where you will follow all the same procedures.

Possible Risks

The risk levels experienced at your appointments will be very minor, similar to those faced when undertaking a medical examination. It is possible that you will experience minor discomfort when blood samples are taken. You may feel the needle passing through the skin or experience minor bruising near the puncture site. There is extremely low risk of infection. Any problems will be treated by the nurse. If you are unhappy with your level of discomfort withdrawal from the study will be advised.

During the study you will be asked on two occasions to drink soymilk which contains a radioactive isotope known as Calcium-45. Calcium-45 is assumed to be uniformly distributed in mineral bone and retained with a biological half life of approximately 165 days. This means that the radiation of the calcium-45 will be reduced by half in approximately 6 months.

This research study involves exposure to a very small amount of radiation in the milk that you will be asked to drink. As part of everyday living, everyone is exposed to naturally occurring background radiation and receives a dose of about 2000 microSieverts each year. The effective dose from the calcium absorption measurements in this study is about 700 microSievert. At this dose level, no harmful effects of radiation have been demonstrated as any effect is too small to measure. The risk is believed to be very low.

Possible Benefits

You will be able to find out how well you absorb calcium from fortified soymilk and fermented fortified milk. From this information, you will be able to obtain advice on how much fortified soymilk you need to consume to meet the calcium RDI (Recommended Daily Intakes)?

Vitamin D level in the blood will also be measured. Vitamin D is very important in assisting in the absorption of calcium from the dietary food sources into the body. This information can help in recommending if vitamin D supplements are required for assisting with optimum calcium absorption from food sources.

If you fit the above criteria, the above information will be of great value to minimise the risk of fracture in the future.

Results of the project

Upon completion of the study, a letter with your personal results and the lay summary of results of the study will be sent.

Confidentiality and Disclosure of Information

The information that you provide will remain confidential. It will be stored in a de-identified (non-personal and specific, that is, no names will be kept) form with the code known to only one researcher (Anne Lise Tang). The code to the information that would allow you to be identified will be destroyed at the completion of the study. The only people who will have access to the data will be the researchers involved in the study. The data will be stored in a locked filing cabinet in a locked room. De-identified data will be transferred to a password protected computer. Printed and electronic data from the study will be kept for seven years after publication of study results. Identifying data will then be destroyed by shredding or electronic deletion. Outcomes/results of the study will be available to the participants and any furthers details about the study can be requested.

Participation is Voluntary

Participation in this research project is voluntary. If you do not wish to take part you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the study at any stage.

Treatment for injuries

In the event that you suffer an injury as a result of participating in this project, hospital care and treatment will be provided by the public health service at no extra cost to you.

Complaints

Should you have any complaints (Research project no. 07013A), you may contact Malar Thiagarajan, Manager, Research Directorate, Southern Health. Telephone (03) 9594 4611.

Ethical Guidelines

This project is being carried out according to the *National Statement on Ethical Conduct in Research Involving Human* (June 1999) produced by the National Health and Medical Research Council of Australia. This project has been approved by the Southern Health Human Research Ethics Committee and Monash University Standing Committee on Ethics in Research involving Humans (SCERH).

Any queries about your participation in this project may be directed to the researchers:

- **Anne Lise Tang ☎ 0401430507 or 03 9919 8221**
- **Dr. Gisela Wilcox ☎ 0413 870 315**
- **Dr. Karen Walker ☎03 9594 4472**
- **A/Professor Lily Stojanovska ☎03 9919 2737**
- **A/Professor Boyd Strauss ☎03 9594 1390,**

APPENDIX C:

PARTICIPANT INFORMATION FOR STUDY

(DETERMINING EQUOL PRODUCTION)

INFORMATION

TO PARTICIPANTS

You are invited to participate in the entitled:

'Determining Equol Production in osteopenic post menopausal women'

Project explanation

Soy-milk consumption is increasing in the western diet largely driven by consumer appreciation via documented research of the potential health benefits of soy isoflavones. Isoflavones are an important group of phytoestrogens. When consumed they have been shown to help decrease the risk of some cancers. They also can reduce menopausal symptoms, help lower serum cholesterol and decrease the risk of osteoporosis.

While there are many soy isoflavones, the one we are interested in for this study is Equol. Equol is produced in the intestine when bacteria break down soy-based food such as soy beans. Interest in Equol has grown in recent years because it is a powerful phytooestrogen (a plant derived compound that may have oestrogen-like effects in the body). Equol can also act as an anti-oxidant protecting the body from damage.

It has recently been shown that some people make equol in the intestine better than others. Moreover, the highest clinical responses to soy protein diets are observed in healthy people who are good 'equol producers'. This new evidence indicates that when we study the effectiveness of soy protein diets in the treatment or prevention of hormone-dependent conditions, we need to identify which participants in our study are good equol producers. This can then explain some of the variability we observe in our data.

We have undertaken two studies comparing the calcium absorption between cows' milk, calcium fortified soymilk and fermented soymilk in osteopenic post menopausal women. Preliminary results show that calcium from calcium fortified soymilk can be absorbed equally well as calcium from cows' milk. However, some participants absorbed both cows' milk and soymilk much better than others. We may well be able to explain this variability if we can determine which participants are 'equol producers' The aim of this study is therefore to determine if participants from the completed study comparing calcium absorption from cows' milk and soymilks were 'equol producers' or not.

We greatly appreciated your interest and time in participating in our two previous studies comparing the calcium absorption from cows' milk to calcium fortified soymilk to fermented calcium fortified soymilk. We would like to invite you to be part of this new study that will determine if you are an 'equol producer' or a 'non equol producer'. Findings from this study are highly relevant to the understanding of the benefits of soymilk consumption and; may also help explain the mechanisms of calcium absorption from calcium fortified soymilk.

Am I suitable to participate in this study?

You are suitable to take part in this study if you are a postmenopausal, osteopenic woman aged between 45 -70 years who:

- Is reasonably healthy.
- Has no strict dietary pattern (e.g. strict vegetarians),
- Is willing to consume calcium fortified soymilk (So Good).

I am not suitable to take part in the study if I :

- Am not post menopausal or post menopausal < 1 year
- have taken hormone therapy (HT) in the past year
- am not osteopenic
- have an allergy to soy
- use prescription or non-prescription drugs such as, antibiotics or medications for conditions such as osteoporosis
- have a serious medical condition such as a bleeding disorder, heart, liver, gastrointestinal (e.g. coeliac disease), metabolic bone or kidney disease.
- regularly consume more than 3 standard alcoholic drinks per day

What will I have to do?

Participant will be asked to drink two glasses (250 mL) of a commercially available soymilk (So Good; Sanitarium Health Foods), one in the morning and one in the evening, on three consecutive days. On the morning of day 3three, you will empty your bladder and then begin to collect all your urine over the next 24 hours. You will be given instructions and a suitable collection container.

What will I gain from participating?

At the end of the study, you will be informed if you are 'equol producer' . This information is valuable since if you are an equol producer you are much more likely to gain benefit from diets high in soy-based foods.

How will the information I give be used?

This study will provide critical information to indicate if being an equol producer enhances calcium absorption from calcium fortified soy-milks. Results from this study may help provide information to the food and nutrition research industry on factors that may optimise calcium absorption from various food sources and may also contribute towards explaining the mechanism of action of soy isoflavones in disease prevention and treatment.

What are the potential risks of participating in this project?

There are no known risks for you in participating in this study, unless you have an allergy to soymilk. You are free to withdraw from the study at any time.

How will this project be conducted?

We have sent you this information sheet because you have expressed interest in participating in this study. Please read the information carefully. If you are suitable for the study and agree to participate you will be asked to sign the consent form. The student researcher will then contact you and arrange a convenient time to deliver soymilk and empty containers for urine collection to you.

You will be asked to drink soymilk on three days and then to collect urine for 24 hours as described above (under "what will I have to do?"

The student researcher will arrange a time to collect the urine from you. Until she collects it, it should be stored in a cool place, preferably in the fridge. The volume of urine you produce in 24 hours will be recorded and then a small sample (50 mL) will be frozen for analysis. S-equol will be measured in this urine by HPLC using electrospray ionization ESI-MS. After analysis, samples will be discarded. Results will tell us how much equol you have in urine which then indicates whether you produce a lot of equol from bacterial fermentation in your intestine. We will send you a letter when the study is complete, giving you your results.

Confidentiality and Disclosure of Information

Any information that you provide will remain confidential. It will be stored in a de-identified (non-personal and specific, that is, no names will be kept) form with the code known to only one researcher (Anne Lise Tang). The code to the information that would allow you to be identified will be destroyed at the completion of the study. The only people who will have access to the data will be the researchers involved in the study. The data will be stored in a locked filing cabinet in a locked room. De-identified data will be transferred to a password protected computer. Printed and electronic data from the study will be kept for seven years after publication of study results. Identifying data will then be destroyed by shredding or electronic deletion. Outcomes/results of the study will be available to the participants and any furthers details about the study can be requested.

Participation is Voluntary

Participation in this research project is voluntary. If participants do not wish to take part you are not obliged to. If participants decide to take part and later change their minds, they are free to withdraw from the study at any stage.

Who is conducting the study?

This research forms one important component of a higher degree (Doctor of Philosophy) investigating the potential health benefits associated with increased calcium absorption in post menopausal women from calcium fortified soymilk. The human dietary intervention study that you are now invited to join will be conducted by a student researcher (Ph.D. candidate at Victoria University), Anne Lise Tang, under the close supervision of experienced academic researchers from the areas of medicine, physiology and food science.

- Assoc. Prof. Lily Stojanovska (*BSc, MSc, PhD*), School of Biomedical and Clinical Sciences, Victoria University - ☎03 9919 2737 – **Principal Researcher**
- Anne Lise Tang (BSc *(Hons)*), School of Biomedical and Clinical Sciences, Victoria University ☎ 9919 8221/0401 430 507 - **Student Researcher**
- Dr Gisela Wilcox (*BMedSc(Hons), MBBS(Hons), FRACP, MAACB, FRCPA)*, Clinical Nutrition & Metabolism Unit, Monash Medical Centre☎ 0413 870 315
- Dr Karen Walker (*BSc(Hons), PhD, MND, APD)*, Nutrition & Dietetics Unit, Monash University Department of Medicine, Monash Medical Centre and Baker Heart Research Institute. ☎03 9076 2991

Any queries about your participation in this project may be directed to the Principal Researcher listed above. If you have any queries or complaints about the way you have been treated, you may contact the Secretary, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 phone (03) 9919 4781.

Procedure for determination of Equol production status.

Thank you for your participation.

Below are the guidelines for the study.

Materials provided:

- 2 X 1L So Good soymilk (please store in refrigerator once open)
- 3 X 2 L empty plastic bottles
- 1 funnel
- Clear plastic bags
- Labels with name and time details
- Information and detail form

This study will be conducted over 3 days and no change in eating habit or diet pattern is required. It is therefore recommended to maintain your usual diet.

Day 1:

1. Drink 250mL (1 glass) of soymilk in the morning.

2. Drink 250mL (1 glass) of soymilk at night or before going to bed.

Day 2:

1. Drink 250mL (1 glass) of soymilk in the morning.

2. Drink 250mL (1 glass) of soymilk at night or before going to bed.

Day 3:

1. Commence 24 hour urine collection in the morning as indicated below.

2. Drink 250mL (1 glass) of soymilk in the morning.

3. Drink 250mL (1 glass) of soymilk at night or before going to bed.

Procedure for 24 hour urine collection:

Note: It is essential that this procedure is followed very carefully.

• Test results are based on the total amount of tested substance excreted by your body over a 24-hour period.

• You should aim to collect every drop of your urine during the specified 24-hour period.

• It does not matter what the volume of the urine is, as long as it represents every drop that you pass. (3 bottles are provided)

• If you have a bowel movement, you must collect the urine separately.

It is **not** recommended to pass urine directly into the container, place the funnel provided into the opening (if required). If necessary collect the urine firstly into a clean, dry, non-metallic object (such as a plastic blue lid container provided) and then pour into the container through the funnel.

Please collect the urine as follows:

1. Begin at the usual time that you wake. The 24 hour collection will be divided into 2 X 12 hour segments.
2. At that time, pass your urine, flush it down the toilet and note the exact time on the container and on the form attached. You will now have an empty bladder and an empty bottle. The collection of urine will start from this time. **Please note both the date and time on the collection bottle label, where it says 'start date and time'.**
3. Collect every drop you pass during the day and night, for 12 hours into the first bottle. Please note time.
4. Then, continue urine collection for another 12 hours into second bottle. And finish the collection by passing urine at exactly the same initial time the next morning. You should add this final specimen to the second bottle. Please note time and date.

5. Keep the sample bottles cool; closed and protected from light between each time you pass urine. It is recommended to keep the bottles in the clear plastic provided and store at cool refrigerated temperature if possible (~4˚C) until collection.

6. The time you pass the last urine specimen should not vary by more than five or ten minutes from the time of starting the collection the previous day. This is the end of the collection. **Please note both the date and time on the collection bottle label, where it says 'finish date and time'.**

7. Please complete the information on the page attached during study.

8. Please check that the cap is firmly screwed onto the container.

After completing your collection

If there is a delay in handing in the collection after completion, the bottle should be kept cool or refrigerated (not frozen). Should you have to, you can store the bottle at a cool room temperature for a day or two.

Please call the student researcher to arrange collection, details as follows:

Anne Lise Tang – Tel no. 0401 430 507

If there is a delay in the collection after completion, the bottle should be kept cool or refrigerated (not frozen).

THANK YOU FOR YOUR PARTICIPATION☺