ENHANCING THE TRANSFORMATION LEVEL OF BIOACTIVE SOY ISOFLAVONES IN SOY-BASED FOODS BY PROBIOTIC ORGANISMS

A thesis submitted for the degree of Doctor of Philosophy

By

THUY THI PHAM

B.E. Hons. (Food Technology)

2010

School of Biomedical and Health Sciences

Faculty of Health, Engineering and Science

Victoria University, Werribee Campus, VIC, Australia

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

I dedicate this PhD thesis to my late elder brother,

Mr. Thai H. Pham, who inspired me learning and wisdom

& to my beloved husband and children who love me unconditionally.

Abstract

The biologically active forms of the isoflavones, aglycones (IA), in soy are reported to have many health benefits and could be considered as a "natural component" to replenish the estrogens in woman at menopausal and post-menopausal age. However, the isoflavones in soy exist principally in isoflavone glycoside (IG) forms, which have lower bioavailability. In order to improve health status of soymilk, it is essential to transform IG to IA.

Initially, pure β-galactosidase and β-glucosidase were utilised for hydrolysing IG to IA in soymilk (SM). The level of hydrolysis ranged from 43.3-77.2% and 86.7-93.0% by various β-galactosidase concentrations and β-glucosidase, respectively.

Six strains of probiotic organisms that produced β-galactosidase and β-glucosidase were used for the biotransformation of IG to IA in soymilk. To enhance the biotransformation level of IG to IA, lactulose and skim milk powder (SMP) were added to SM. The presence of lactulose in the medium enhanced the biotransformation level of IG to IA by *Lactobacillus* up to 21.9%. In particular, *L. acidophilus* 4461 biotransformed 88.8% IG to IA, the highest level recorded, in SM supplemented with lactulose 0.05% (w/w) (SML). The biotransformation of IG to IA was also enhanced significantly by 6.8 – 17.1% and 12.8 – 13.5% in SML by *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099, respectively. Similarly, the biotransformation level of IG to IA in SM supplemented with SMP (SSM) ranged from 81.4 to 85.1%, which was 13.9 to 19.0% higher than that for SM. The levels of biotransformation were 84.0% and 85.4% for *Bifidobacterium animalis* subsp. *lactis* bb12 and *B. longum* 20099, respectively, compared to 74.3% and 72.8% for the SM. The supplementation with lactulose or SMP also significantly ($P<0.05$) improved the viability of the probiotic organisms.

Finally, soy protein isolate (SPI) (4.0%, v/w) was supplemented to the yogurt mix to increase ($P<0.05$) IA concentration in yogurt (SY). The supplementation significantly increased the lactose metabolism by the yogurt starter including *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (Lb 11842) and *Streptococcus thermophilus*

ST 1342 (*S. thermophilus* 1342) during the fermentation process by 4.7%. The viability of both Lb 11842 and *S. thermophilus* 1342 in SY remained high during the storage period (8.11- 8.84 log CFU/g). The starter transformed 72.8% of IG to IA, increasing the IA content from 1.35 to 15.01 mg/100 g sample.

Declaration

I, Thuy Thi PHAM, declare that the PhD thesis entitled "***Enhancing the transformation level of bioactive soy isoflavones in soy-based foods by probiotic organisms***" 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.

April, 2010

Thuy Thi PHAM

School of Biomedical and Health Science

Werribee Campus, Victoria University

Melbourne, Victoria, Australia

Acknowledgement

Firstly, the supervision, advice and guidance provided during my project by Professor Nagendra P. Shah, at School of Biomedical and Health Sciences is greatly appreciated with thanks.

I am thankful to the Faculty of Health, Engineering and Sciences and the School of Biomedical and Health Sciences, Victoria University for offering me the PhD scholarship and the financially supporting attendance to conferences.

I wish to thank my hard-working laboratory manager, Mr. Dale Tomlinson and all lab technicians especially Mr. Joseph Pelle and Mrs. Min Thi Nguyen for the technical assistance. Their enthusiasm and expertise enabled me to use modern and complicated equipments such as HPLC system in order to analyse my samples.

I am also grateful to my friends, Osaana Donkor, Daniel Otieno, Muditha Dissanayake, Hongli Wu, Lata Ramchandran, Hien Van Dao, Liyana Arachchi Rupika Herath and all other lab mates for their friendship and support.

Finally, the most special and important thank goes to each member of my family including my beloved husband, Dr Hai P. Le and my two kids. My husband has continuously supported me and his contribution to my thesis is something I am forever indebted. My beautiful daughter Bao Han, who now is able to present a 5-minute talk about my PhD project and my little son Duy Anh who was born during my PhD course, together have helped me discovering my unexplored internal strength and energy of as a Mum of two could have to overcome all the obstructions.

Thuy Thi PHAM

Publication and Awards

Journal Papers

1. Pham, T. T., & Shah, N. P. (2009). A review of soy isoflavones – Controversy of the bioavailability, transformation and health Effects. **Critical Reviews in Food Science and Nutrition**, Under review.
2. Pham, T. T., & Shah, N. P. (2009a). Hydrolysis of isoflavone glycosides in soymilk by β-galactosidase and β-glucosidase. **Journal of Food Biochemistry**, 33, 38-60.
3. Pham, T. T., & Shah, N. P. (2009b). Performance of starter in yogurt supplemented with soy protein isolate and biotransformation of isoflavones during storage period. **Journal of Food Science**, 74, M190-M195.
4. Pham, T. T., & Shah, N. P. (2008d). Effect of lactulose supplementation on the growth of bifidobacteria and biotransformation of isoflavone glycosides to isoflavone aglycones in soymilk. **Journal of Agricultural and Food Chemistry**, 56, 4703-4709.
5. Pham, T. T., & Shah, N. P. (2008c). Skim milk powder supplementation affects lactose utilization, microbial survival and biotransformation of isoflavone glycosides to isoflavone aglycones in soymilk by *Lactobacillus*. **Food Microbiology**, 25, 653-661.
6. Pham, T. T., & Shah, N. P. (2008a). Fermentation of reconstituted skim milk supplemented with soy protein isolate by probiotic organisms. **Journal of Food Science**, 73, M62-M66
7. Pham, T. T., & Shah, N. P. (2008b). Effect of lactulose on biotransformation of isoflavone glycosides to aglycones in soymilk by lactobacilli. **Journal of Food Science**, 73, M158-M165.

8. Pham, T. T., & Shah, N. P. (2007). Biotransformation of isoflavone glycosides by *Bifidobacterium animalis* in soymilk supplemented with skim milk powder. **Journal of Food Science**, 72, M316 -M324.

Conference Papers

1. Pham, T. T., & Shah, N. P. (2009). Poster presentation. Enhancing nutritional value of soy yogurt supplemented with skim milk and probiotic organisms. **42nd Annual AIFST Convention**, 13-16 July 2009 Brisbane, Australia.
2. Pham, T. T., & Shah, N. P (2009). Oral and Poster presentation. Role of probiotic organisms in transformation of inactive isoflavone to bioactive forms in soymilk. **13th Australian Food Microbiology,** 24-26 March 2009, Melbourne, Australia.
3. Pham, T. T., & Shah, N. P. (2009). Oral Presentation. Increasing the nutritional values of soy yogurt supplementation with skim milk powder. Full Proceeding Paper. **11th Government Food Analysts Conference**, 22-24 Feb 2009, Melbourne, Australia.
4. Pham, T. T., & Shah, N. P. (2009). Oral Presentation. Effects of skim milk powder supplementation to soy yogurts on biotransformation of isoflavone glycosides to biologically active forms during storage. ICAFNS 2009. Full Proceeding Paper. **International Conference on Agricultural, Food and Nutritional Sciences**, January 28-30, 2009, Dubai, United Arab Emirates. ISSN 2070-3740.
5. Pham, T. T., & Shah, N. P. (2008). Poster Presentation. Effect of lactulose on the biotransformation of isoflavone glycosides to isoflavone aglycones by probiotic organisms in soymilk. **41st Anniversary AIFST Convention**, 21 – 24, July 2008, Sydney, Australia.
6. Pham, T. T., & Shah, N. P. (2007). Poster Presentation. Biotransformation of isoflavone glycosides to isoflavone aglycones in soymilk supplemented with lactulose by *Bifidobacterium animalis* subsp. *lactis* bb12. **International Conference: From Bioscience to Biotechnology and Bio-industry**, 18-19 December, 2007, Hanoi, Vietnam.

7. Pham, T. T., & Shah, N. P. (2007). Poster Presentation. Enhancing the biotransformation of isoflavone glycosides to bioactive forms in soymilk by probiotic organisms. **40th Anniversary AIFST Convention**, 24-27 June 2007, Melbourne, Australia.

Awards and Grants

1. Ranked number one of the International Postgraduate Research Scholarship grant for 3 years PhD course
2. A scholarship holder of a scholarship from Faculty of Health, Engineering and Science, Victoria University, Australia
3. Secomb Conference and Travel Fund Award from Victoria University for my oral presentation at International Conference on Agricultural, Food and Nutritional Sciences. Dubai, United Arab Emirates, January 28-30, 2009

Table of Contents

List of Figures

List of Tables

List of Abbreviations

2-de-O-DMA: 1-(2,4-dihydrobenzoyl)-1-(4-hydroxyphenyl)ethylene

4-HP-2-PA: 4-hydroxyphenyl propionic acid

6'- OH-DMA: 6'-hydroxy-O-demethylangolensin

CIs: Confidence Intervals

ERα: Estrogen Receptor α

ERβ: Estrogen Receptor β

HDLC: High Density Lipoprotein Cholesterol

HPLC: High Performance Liquid Chromatography

IA: Isoflavone Aglycones

IG: Isoflavone Glycosides

LAB: Lactic Acid Bacteria

Lb 11842: *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842:

LDLC: Low Density Lipoprotein Cholesterol

Lp: Lipoprotein

ORs: Estimate Odds ratios

p-NPG: p-nitrophenyl-β-D glucopyranoside

PSA: Serum (prostate-specific antigen)

RSM: Reconstituted Skim Milk Powder

SERMs: Nature's Selective Estrogen Receptors

SML: Soymilk Supplemented With Lactulose

SHBG: Sex Hormone-Binding Globulin

SI: Soy Isoflavones

SM: Soymilk

SMP: Skim Milk Powder

SPI: Soy Protein Isolate

SSM: Soymilk Prepared Soy Protein Isolate Supplemented With Skim Milk Powder

***S. thermophilus* 1342**: *Streptococcus thermophilus* ST 1342:

THB: Trihydroxybenzene

TPC: Total Plasma Cholesterol

USY: Yogurt without Supplementation with SPI

VLDLC: Very Low Density Lipoprotein Cholesterol.

Chapter 1.0

Introduction

Isoflavones are classified as a flavonoid group of phytoestrogens and are promising natural substances as they provide many health benefits, such as relief of menopausal symptoms, improvement in bone health and lowering the incidence of cardiovascular diseases (Hughes et al., 2003). Especially, isoflavone compounds are able to replenish the estrogens in women at menopausal and post-menopausal age without side effects.

In nature, isoflavones are found abundantly in legume family (fabaceae or leguminosae). In the legume family, the main edible source of isoflavone compounds is soybean (*Glycine max*). However, in nature as well as in non-fermented soy food products, isoflavones predominantly exist as β-glycoside conjugates ranging from 83.9 to 98.4% (King & Bignell, 2000). Compared to glycoside isoflavone glycosides (IG), non β-glycoside conjugated isoflavone compounds (isoflavone aglycones, IA) including daidzein, genistein, glycitein, biochanin A and formononetin are more bioavailable (King, 1998; Piskula, Yamakoshi, & Iwai, 1999; Setchell et al., 2001). To obtain health benefits, IA intake required is approximately 40 mg/d (Malnig & Brown, 2007). Although IG is thought to be hydrolysed to IA in our gastro-intestinal tract, it still remains unclear as to how much IG would be transformed to IA. Until now, gut microflora are thought to play a key role in the transformation of IG to IA. Consequently, the biotransformation level of IG to IA strongly depends on each individual such as diet, medication, sex, location and so on (Rowland et al., 1999; Slavin, Karr, Hutchins, & Lampe, 1998; Uehara et al., 2001).

Accordingly, food products containing a considerable amount of IA are a novel trend in the food industry. To transform IG to IA, several methods have been used. The most popular method is the use of lactic acid bacteria (LAB) or probiotic organisms for biotransformation of IG to IA (Tsangalis et al. 2002; Chien et al., 2006, Otieno et al. 2006a; Farnworth et al., 2007; Donkor et al., 2007). However, the biotransformation level of IG to IA is normally low (Chien et al., 2006). Accordingly, the IA content in the final product is also low. For instance, *L. acidophilus* biotransformed only 5.3% of the total IG to IA in fermented soymilk in 32 h at 37 ^{o}C (Chien et al., 2006). Therefore, a research question was raised: How to enhance the transformation of IG to IA in order to provide healthy soy-based products with a moderate concentration of IA. If the microbial methods is utilised, it is essential to enhance the capability of the microorganisms in the biotransformation of IG to IA. However, so far, no research has been carried out to enhance the biotransformation of IG to IA. Initially, for enhancing the biotransformation

of IG to IA in fermented soymilk by probiotic organisms, initially, enzymes which are responsible for the biotransformation were investigated.

In this study, soymilk was made from soy protein isolate (SPI) as it consistently contains a good amount of isoflavones. SPI is considered a perfect source of protein and commonly utilised in food industry (Riaz, 2006). Therefore, SPI was selected to be a reliable material for the transformation of IG to IA.

The specific aims of this project were:

- To assess whether pure β-galactosidase could hydrolyse IG to IA and to examine the effectiveness of pure β-glucosidase on the biotransformation of isoflavone glycosides to aglycones in soymilk,
- To investigate the effect of lactulose supplementation on the growth of *Lactobacillus* and bifidobacteria and their biotransformation ability of IG to IA in fermented soymilk,
- To investigate lactose utilisation by *Lactobacillus* and bifidobacteria, their survival, and the biotransformation of IG to IA in soymilk supplemented with skim milk powder (SMP), and
- To examine the influence of the supplementation with soy protein isolate on the performance of yogurt starter including *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* on lactose utilisation, organic acids production, survival of the starter organisms and the biotransformation of IG to IA in soy yogurt by the yogurt starter during the storage period of 28 days at 4 ^{o}C.

Chapter 2.0, the literature review, provides a critical review of the soy isoflavones: the transformation ways of IG to IA and the side effects on human health to give the readers the whole picture of the research and studies about soy isoflavones. Chapter 3.0 presents the hydrolysis of IG to IA by two enzymes including β-glucosidase (EC 3.2.1.21) and β-galactosidase (EC 3.2.1.23). It was shown that both of the two enzymes are able to cleave the β-glucosidic bond between IA and the β-glycoside moiety in IG molecule. Therefore, in order to enhance the biotransformation of IG to IA, the activity of the two enzymes needed to be improved. To stimulate the enzyme activities, lactulose and skim milk powder were supplemented to soymilk individually which were fermented by *L. acidophilus* 4461, *L. acidophilus* 4962, *L. casei* 290, *L. casei* 2607, *B. animalis* subsp

lactis bb12 and *B. longum* 20099. These are presented in chapter 4.0 and chapter 5.0, respectively. Chapter 6.0 presents the application of high IA containing yogurt in food industry, which is the biotransformation of isoflavones and the performance of starter in yogurt supplemented with soy protein isolate during storage period. Chapter 7.0 concludes the significant findings of the project and suggests the future research directions that need to be carried out to complete the missing puzzles about soy isoflavones. All references are included in Chapter 8.0.

Chapter 2.0

Literature Review

This chapter has been submitted as:

Pham, T. T., & Shah, N. P. A Review of Soy Isoflavones – Controversy of the Bioavailability, Transformation and Health Effects. **Critical Reviews in Food Science and Nutrition.** (Under review)

2.1 What are isoflavones?

Isoflavone compounds are phytochemicals, which are a class of flavonoids having polyphenolic structure. As they possess a weak estrogenic effect, they are also classified as phytoestrogens. Figure 2.1 shows the relationship of isoflavone compounds with other phytoestrogen compounds.

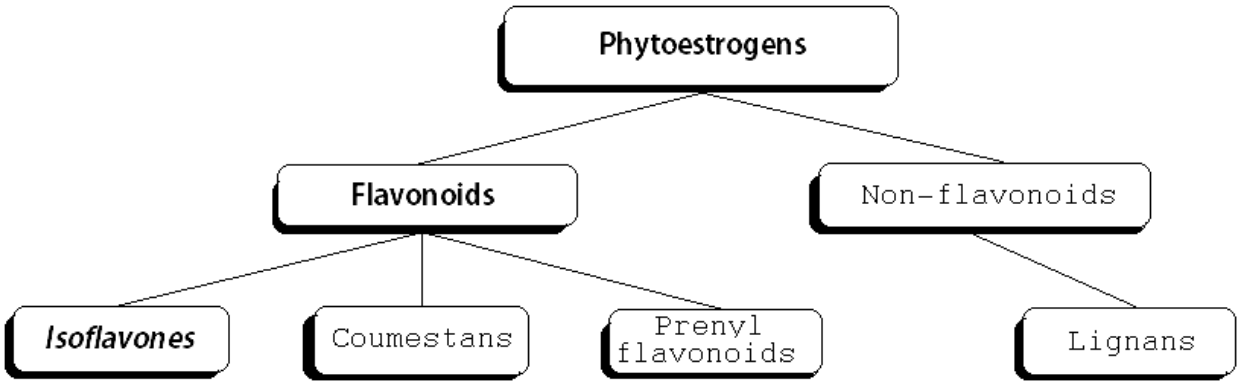


Figure 2.1 Phytoestrogen compounds

Adapted from Hughes et al. (2003)

2.1.1 Isoflavone forms in nature

In nature, isoflavone compounds are present in two main groups as shown in Table 2.1. Group 1, isoflavone aglycones (IA), is not conjugated to a β-glycoside. Group 2 includes three sub-classes which conjugate to the β-glycoside, isoflavone glycosides (IG). The structure of isoflavone compounds is shown in Figure. 2.2.

2.1.2 The sources of isoflavone in nature and food products

Isoflavones are commonly found in legume family (fabaceae or leguminosae). In the legume family, the two main sources of isoflavone compounds are soybean (*Glycine max*) and red clover (*Trifolium pratense*) in which they occur mainly in glycoside forms (Figure 2.2). Interestingly, they are also found in a trace amount in cow milk, breast milk, fruits and grains (Knight, Eden, Huang, & Waring, 1998; Liggins et al., 2000a, b; Slavine, 1996). Accordingly, the soy food products are rich sources of isoflavones.

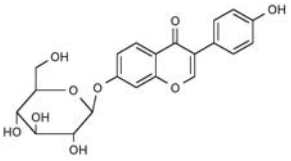
Table 2.1 Isoflavone compounds found in nature and foodstuffs

Isoflavone compounds		Formula	Molecular weight
Group1: Isoflavone aglycones			
Un-conjugated	Daidzein	$C_{15}H_{10}O_4$	254
	Glycitein	$C_{16}H_{12}O_5$	284
	Genistein	$C_{15}H_{10}O_5$	270
	Biochanin A	$C_{16}H_{12}O_5$	284
	Formononetin	$C_{16}H_{12}O_4$	268
Group 2: Isoflavone glycosides			
Conjugated to glucose	Daidzin	$C_{21}H_{20}O_9$	416
	Glycitin	$C_{22}H_{22}O_{10}$	446
	Genistin	$C_{21}H_{20}O_{10}$	432
	Ononin (Biochanin A glucoside)	$C_{22}H_{22}O_{10}$	446
	Sissotrin (Formonenin glucoside)	$C_{22}H_{22}O_{10}$	446
Conjugated to acetyl glycosides	Acetyl daidzin	$C_{23}H_{22}O_{10}$	458
	Acetyl glycitin	$C_{24}H_{24}O_{11}$	488
	Acetyl genistin	$C_{23}H_{22}O_{11}$	474
Conjugated to malonyl glycosides	Malonyl daidzin	$C_{24}H_{22}O_{12}$	506
	Malonyl glycitin	$C_{25}H_{24}O_{13}$	532
	Malonyl genistin	$C_{24}H_{22}O_{13}$	518

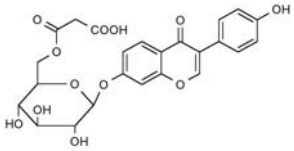
Table 2.2 presents the isoflavone concentration in soy food and other food products. Recently, isoflavone compounds have been found in meat products as well, since soy, especially soy protein isolate (SPI), is usually added as a food additive (Vranova, 2005).

2.1.3 Soy isoflavones

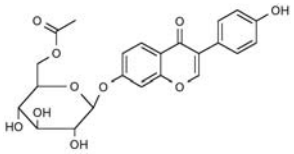
As shown in the Table 2.2, the main and the richest source of isoflavone from edible source in nature is soybean. Soybeans were first recognised to contain isoflavones more than 70 years ago, when genistin was isolated in crystalline form from a 90% methanol extract of soybeans and acid hydrolysis (Walter, 1941). Nowadays, soy bean and soy product are considered a global food, although they are still new food for many people in the Western society. However, in Asia, soy bean has been consumed for almost 5000 years. Soybeans and soy foods were officially introduced to the Western society at the beginning of the twentieth century. Some soy foods, such as soy sauce and soymilk, have been accepted by Westerners for the past several decades. Recently, many new products from soy have been introduced such as soy ice cream, soy yogurt, veggie burgers, soy sausage, and soy flour pancakes. In 1936, SPI was first introduced by Percy Lavon Julian, an organic chemist. Soy protein isolate contains up to 85-90% of proteins and is considered the perfect source of proteins as it has the highest score of protein digestibility.



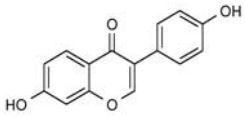
Daidzin



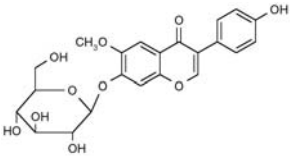
Malonyl daidzin



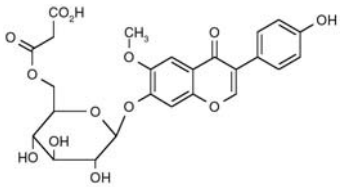
Acetyl daidzin



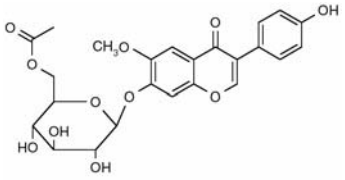
Daidzein



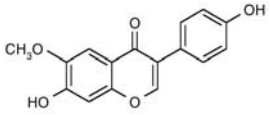
Glycitin



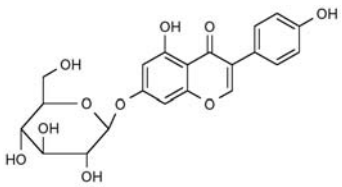
Malonyl glycitin



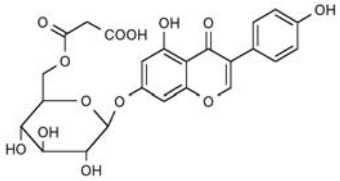
Acetyl glycitin



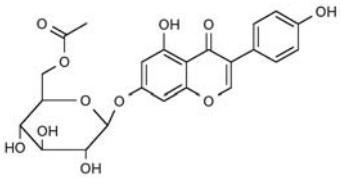
Glycitein



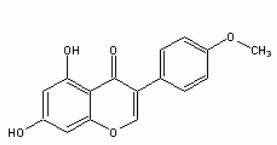
Genistin



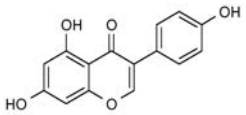
Malonyl genistin



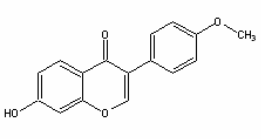
Acetyl genistin



Biochanin A



Genistein



Formononetin

Figure 2.2 Chemical structures of isoflavones

Accepted from Hughes et al. (2003)

Table 2.2 Isoflavone compounds (IG and IA) in food products

Food products	Isoflavone aglycones (mg/100g)	Isoflavone glycosides (mg/100g)	References
Soy and Soy Foods			
Soybean	8.0[a]	161.0[a]	(King & Bignell, 2000)
Soy flour	4.5[a]	196.9[a]	(Wang & Murphy, 1994)
Soya and linseed bread	2.9[a]	16.9[a]	(King & Bignell, 2000)
Canned bean	2.9[a]	76.6[a]	(King & Bignell, 2000)
Soya flakes	5.7[a]	170.3[a]	(King & Bignell, 2000)
Soya flour	2.2[a]	185.8[a]	(King & Bignell, 2000)
Soya grits	2.7[a]	163.3[a]	(King & Bignell, 2000)
Soya milk	1.4[a]	22.3[a]	(King & Bignell, 2000)
Soy protein isolate A	25.2[a]	36.9[a]	(Wang & Murphy, 1994)
Soy protein isolate B	7.2[a]	91.5[a]	(Wang & Murphy, 1994)
Tofu	1.8[a]	9.4[a]	(King & Bignell, 2000)
Roast soybean	16.0[a]	249.0[a]	(Wang & Murphy, 1994)
Fermented bean curd	ND	38.9[a]	(Wang & Murphy, 1994)
Soya sauce	0.9[a]	0.3[a]	(King & Bignell, 2000)
Tempeh	35.4[a]	24.8[a]	(Wang & Murphy, 1994)
Miso	14.2[a]	24.7[a]	(Wang & Murphy, 1994)
Fruits and Vegetables			
Apple and apple products	ND	ND	(Liggins et al., 2000a)
Avocado	ND	ND	(Liggins et al., 2000a)
Current	22.4[a]	NA	(Liggins et al., 2000a)
Figs	0.5[a]	NA	(Liggins et al., 2000a)
Mango	0.7[a]	NA	(Liggins et al., 2000a)
Melon, honeydew	0.25[a]	NA	(Liggins et al., 2000a)
Passion fruit	1.74[a]	NA	(Liggins et al., 2000a)
Plum	0.75[a]	NA	(Liggins et al., 2000a)
Asparagus	0.1[a]	NA	(Liggins et al., 2000b)
Beetroot	ND	NA	(Liggins et al., 2000b)
Broccoli	0.1[a]	NA	(Liggins et al., 2000b)
Mushroom	0.2[a]	NA	(Liggins et al., 2000b)
Potatoes	0.8[a]	NA	(Liggins et al., 2000b)
Brown rice	ND	ND	(Mazur, Duke, Wahala, & Adlercreutz, 1998)
Meat and Animal Products			
Cow milk	0.001 - 0.03[c]		(Knight et al., 1998)
Breast milk			(Hughes et al., 2003)
Omnivorous mum (n=14)	0-0.2 [b]		
Vegetarian mum (n=14)	0.1-1.0[b]		
Vegan mum (n=11)	0.2-3.2[b]		
Egg and egg yolk	Trace	Trace	(Saitoh, Sato, Harada, & Matsuda, 2004)

ND: not detected according to the determination method of the cited paper, NA: Not achieved according to the cited paper, [a]: Authors recalculated from the cited reference. [b]: data was presented in µg/100mg. [c]: mg/L

Furthermore, SPI can perform many functions such as an emulsifier in a huge range of food products from biscuits, meat products and dairy products (Liu, 2004; Riaz, 2006; Snyder & Kwon, 1987; Vranova, 2005). Also, SPI contains a considerable amount of isoflavones at approximately 150 mg/100g of dry matter (King & Bignell, 2000). Hence,

soy isoflavones (SI) are also present in a wide range of food products and are consumed by people all over the world.

Among five IA, biochanin A and formononetin and their glycosides are not identified in soy, soy products and germinated soybean but they exist in red clover (King & Bignell, 2000; Nakamura et al., 2001; Tsunoda, Pomeroy, & Nestel, 2002;. King & Bignell (2000) stated that the main isoflavone compounds in soybean were daidzin, genistin, malonyl daidzin and malonyl genistin while the most isoflavone components in SPI was malonyl genistin.

2.2 The bioavailability of soy isoflavones

To understand the bioavailability, an understanding of transformation, absorption and metabolisms of SI is required.

2.2.1 Transformation of IG to IA and absorption of SI in human

The transformation and absorption of SI in human body is still not fully investigated and understood. Isoflavone compounds in soy foods especially in non-fermented products are predominantly present in IG forms (Table 2.2). It has been proven that IG does not cross the intestinal wall of healthy humans fed either the pure compounds or a soy food (Setchell et al., 2002). Also, IA are more readily absorbed than IG due to their higher hydrophobicity and lower molecular weight (Hughes et al., 2003). It is thought that IA are absorbed directly from the gastrointestinal tract, whereas IG require cleavage to IA prior to absorption and the cleavage of the β-glycoside conjugates would not occur until IG reach the microflora in the large intestine (Barnes, 1995; Day et al., 1998). However, several studies have reported contradicting findings. Firstly, in the gastrointestinal tract, IG were converted to IA by the salivary enzyme. Up to 70% of genistin could be converted to genistein in a period of 90 min; however this may not be applicable in a real life situation (Allred et al., 2001). Secondly, the hydrolysis of IG can occur in the stomach (Kelly et al., 1993). On contrast, IG were proven to be easily dissolved but still stable in the acidic condition (pH 2.0) of the rat stomach (Piskula et al., 1999). Then, small intestine and liver cell-free extract also was able to de-glycoside most of daidzin and genistin to their aglycones in 90 min (Day et al., 1998). The experiment was also carried out with the extract of small intestine and liver tissues which may not be appropriate in the real life.

However, most scientists agree that in the large intestine, gut microflora play a key role in the transformation of IG to IA (Barnes et al., 1996; Chien et al., 2006; Donkor & Shah, 2008; Farnworth et al., 2007; Kneifel, Rajal, & Kulbe, 2000; Otieno, Ashston & Shah, 2006a; Tsangalis et al., 2002; Xu et al., 1995). Obviously, since the linkage of IA and their β-glycoside moieties is β-glucosidic bond, the gut microorganisms such as lactobacilli, bifidobacteria and bacteroides, *Enterococcus*, *Streptococcus*, and *Weissella* are able to generate β-glucosidase (EC 3.2.1.21) to hydrolyse the β-glucosidic bond (Chun et al., 2007). However, the level of the transformation of IG to IA and IA metabolism by the gut microflora strongly depends on each individual such as age, sex, location, and diet (Frankenfeld et al., 2005; Hughes et al., 2003).

There is still a disagreement about the absorption of IA and IG. In the study of Setchell et al. (2002), IG were not detected in plasma suggesting that IG were not able to be absorbed through the human gut wall. In the study of Richelle et al. (2002), similar levels of plasma and urine pharmacokinetics were observed for the IA and IG enriched drinks.

In agreement with Richelle et al. (2002), the absorption of daidzein and genistein and their glycosides (i.g daidzin and genistin) of 14 subjects was similar in the study of Tsunoda et al. (2002). The reason of the results in Richelle et al. (2002) may due to the capacity of transformation of IG to IA by the subjects. If all the subjects in the study of Richelle et al. (2002) had a high transformation of IG to IA level by gut flora, the concentration of IA in their plasma after the consumption of IG or IA enriched beverage could not be significantly different. However, in the study of Izumi et al. (2000), IA including daidzein and genistein were absorbed much faster and in higher amount than their IG counterparts. Finally, those isoflavones that are not absorbed are excreted in the un-conjugated form in the faeces (Adlercreutz et al., 1995). The transformation of IG to IA of SI in human gastrointestinal tract is summarised in Figure 2.3.

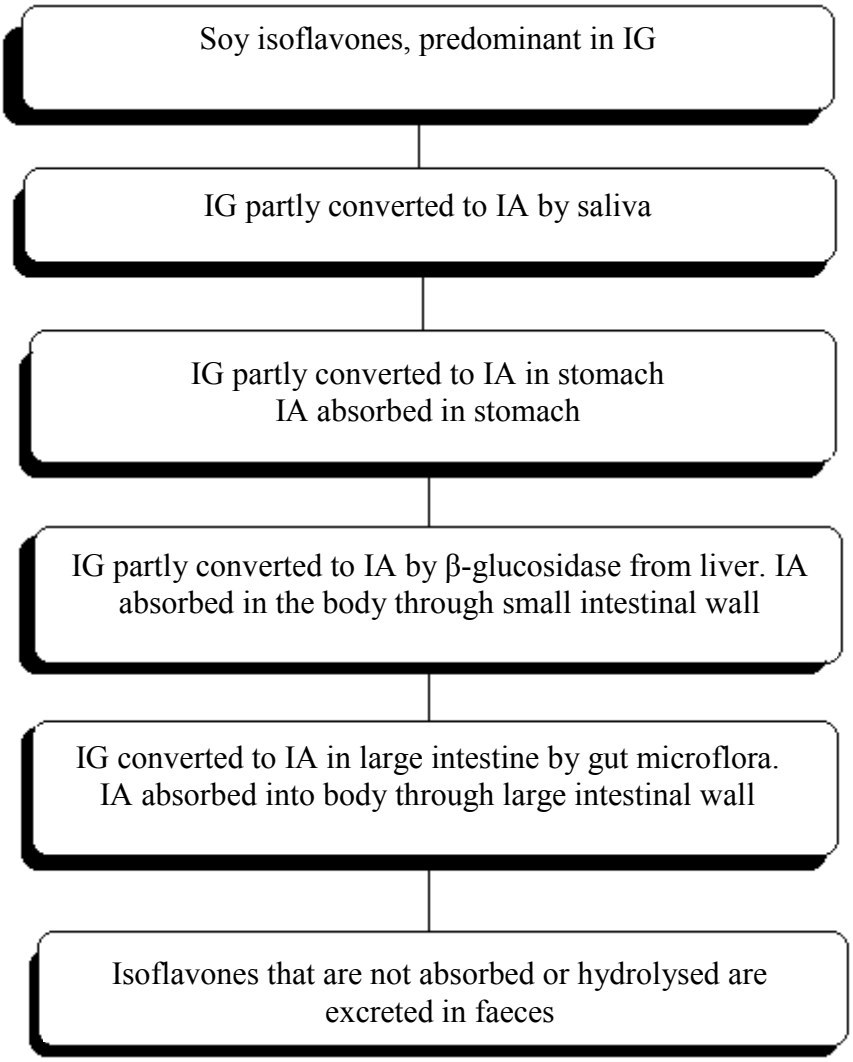


Figure 2.3 The transformation and absorption of soy isoflavones in human

2.2.2 Further metabolism of SI by gut microflora

After absorption through the gut wall in aglycone forms, further metabolism of IA including daidzein and genistein was investigated. However, there was little knowledge about further metabolism of glycitein since its proportion in SI is very minor (King & Bignell, 2000). Daidzein it partially converted to glucuronide and sulphate conjugates by enzymes in the liver or gut microflora before entering the peripheral circulation. These conjugates can be excreted back into the gut from the liver via the bile duct (enterohepatic circulation) where they can be deconjugated by gut microfloral enzymes. They may then be re-absorbed or further transformed in the gut and absorbed since some gut bacteria also possess arylsulfatase activity, which can liberate aglycones from conjugates excreted in

the bile and render them available for re-absorption (Hughes et al., 2003). In human urine, 86% and 75% of genistein and daidzein, respectively, was excreted in glucuronide conjugated forms (Cimino, Shelnutt, Ronis, & Badger, 1999). Then, daidzein and genistein could be degraded further by gut microflora. Figure 2.4 presents the transformation of daidzein to equol.

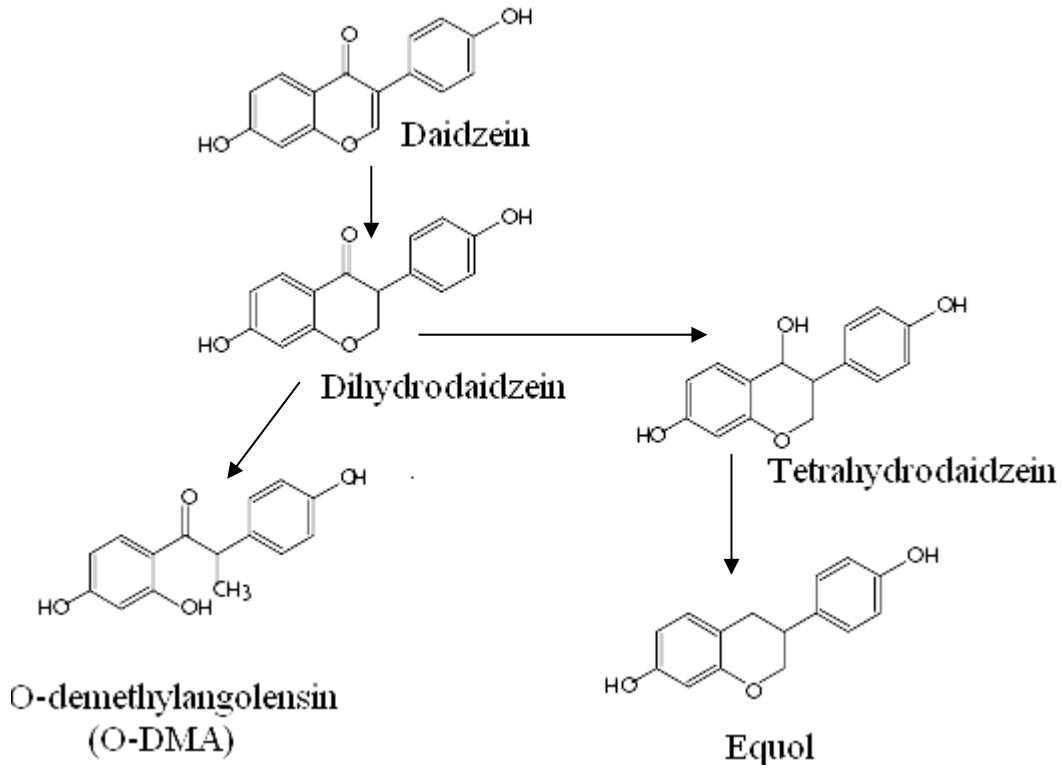


Figure 2.4 Metabolism of daidzein to equol

Adapted from Joannoua et al. (1995)

Firstly, daidzein ($C_{15}H_{10}O_4$) is converted to dihydrodaidzein ($C_{15}H_{12}O_4$) then tetrahydrodaidzein ($C_{15}H_{14}O_4$) and finally to equol ($C_{15}H_{14}O_3$). Dihydrodaidzein is also converted to O-demethylangolensin (O-DMA: $C_{15}H_{12}O_4$).

Similarly, genistein is also reduced to 6'-hydroxy-O-demethylangolensin (6'- OH-DMA). Figure 2.5 shows the metabolism of genistein to 6'- OH-DMA (Joannoua et al., 1995). After that, the 6'- OH-DMA could be further metabolised to trihydroxybenzene (THB) and 4-hydroxyphenyl-2-propionic acid (4-HP-2-PA) (Coldham et al., 2002). Therefore, according to the study of Coldham et al. (2002) the final metabolites of genistein must be

4-HP-2-PA and THB rather than 4- ethylphenol as in the studies of Barnes et al., (1998) and King & Bursill (1998) have reported.

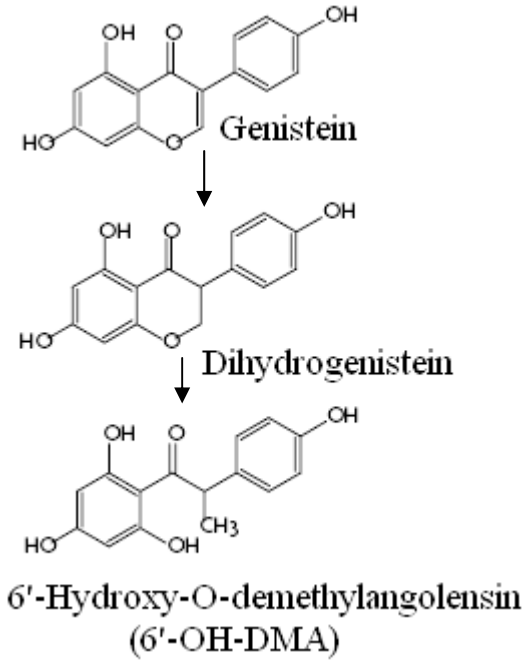


Figure 2.5 Metabolism of genistein to 6'- OH-DMA

Adapted from Joannoua et al. (1995)

2.2.3 Distribution of SI in human body

Isoflavones have been detected in a number of body fluids such as urine, plasma, faeces, prostatic fluid, semen, bile, saliva, breast milk, breast aspirate and cyst fluid. The major isoflavones and their metabolites detected in the blood and urine of humans and animals are daidzein, genistein, equol and 6'- OH-DMA (Adlercreutz et al., 1995). Isoflavones are also found in rat mammary glands, thyroid, liver, prostate, testes, ovary and uterus (Chang et al., 2000).

2.2.4 Factors affecting the metabolism of SI

The metabolism of SI in human body strongly depends on each individual. In the study of Rowland et al. (1999) on 23 subjects that consumed exactly the same amount of isoflavones at 56 mg/day for 7 days, their urinary equol concentration ranged from 30 to 12,000 nmol equol/day. Fifteen out of 23 subjects were defined to be poor equol

producers. The factors described below are reported to affect the metabolism of SI in human body.

2.2.4.1 Diet

Studies in humans have shown that diet can influence metabolism of SI mediated by the gut microflora. For example, consumption of less fat and more carbohydrate, as a proportion of total energy intake, has been correlated with greater equol production, particularly in women. The study of Slavin et al. (1998) suggests that the fermentable carbohydrate content of the diet may be an important variable in determining equol production. Higher intakes of dietary fibre may promote the growth of bacterial populations responsible for equol production in the colon (Uehara et al., 2001). Moreover, the absorption of genistein was significantly enhanced in rats fed with fructo-oligosaccharides than those in controls, but the absorption of daidzein did not differ (Uehara et al., 2001). The effect of diet on the metabolism of SI, both from the influence on gut microflora and differences in hepatic enzyme activities may in part explain any ethnic differences in the metabolic, and perhaps biological response to isoflavones (Hughes et al., 2003).

2.2.4.2 Gender

Gender has been reported to affect the metabolism and excretion of isoflavones. During a one-month trial in which soymilk was ingested (80-210 mg each of genistein and daidzein/day) the excretion half-life progressively shortened in women but progressively lengthened in men throughout the trial (Lu & Anderson, 1998). Furthermore, dihydrogenistein was identified as the major product in the faeces of female rats at 48 hours in contrast to the male rats where 4-HP-2-PA was identified as the major metabolite (Coldham et al., 1999).

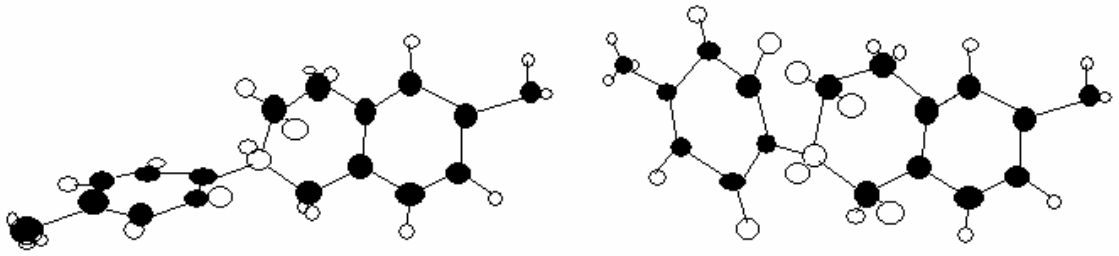
2.2.4.3 Other factors

As the gut microflora play a key role in the metabolism of SI, the factors such as antibiotic use, bowel disease, stress, gut motility, gastric pH, mucins secretion, bile secretion, and intestinal transit time are likely to affect the SI metabolism (Rowland et al., 1999).

2.2.5 The bioavailability of soy isoflavones

Bioavailability is a measurement of the extent of a therapeutically active drug that reaches the systemic circulation and is available at the site of action. After summarising the transformation and absorption of SI of many previous studies, clearly IA are more bioavailable than their IG counterparts (King, 1998; Piskula et al., 1999; Setchell et al., 2001; Hendrich, 2002; Richelle et al., 2002; Setchell et al., 2002). Among IA group, daidzein is considered to have the strongest bioavailability (Lu & Anderson, 1998; Xu et al., 1994). This is in agreement with the study of King (1998), in which based on plasma and urine, daidzein was shown to be more bioavailable than genistein in rat. In contrast, Setchell et al. (2001) stated that genistein was present in a much greater amount than that administered at the same level. The lower concentration of daidzein in plasma is not due to their lower availability but because daidzein is more widely distributed within the body (Dixon, 2004). Biochanin A and formononetin are rapidly demethylated following ingestion to genistein and daidzein, respectively, by either gut microflora or hepatic enzymes (Setchell et al., 2001).

Among the soy isoflavone metabolites, equol is significantly more estrogenic and may be largely responsible for the physiological effects of isoflavone intake (Lu & Anderson, 1998). Equol has two distinct enantiomeric isomers, S-equol and R-equol as shown in Figure 2.6.



S-Equol **R-Equol**

Figure 2.6 Chemical structures of S and R-equol

Adapted from Setchell et al. (2005)

Between the two optical isomers, S-equol showed a strong affinity for estrogen receptor β (ERβ) while R-equol showed a poor capacity in binding to both ERβ and estrogen receptor

α (ERα). For that reason, R-equol is classified as having very weak estrogenic activity. Furthermore, faecal bacteria are able to produce S-equol as the principal metabolite of the daidzein (Setchell et al., 2005). However, in several studies, excretion of equol only occurred in approximately 35% of cases, regardless of gender (Slavin et al., 1998). Unfortunately, there is very little information about the bioavailability of glycitein to human. The pharmacokinetics of glycitin and glycitein still remain unknown. In the study of Setchell et al. (2001), when pure glycitin was ingested as a single-bolus dose by one healthy man, its aglycone form, glycitein, rapidly appeared in the plasma with peak plasma concentrations occurring 4 h after ingestion.

2.3 Transformation of IG to IA

Since IA are more bioavailabe than IG, the transformation of IG to IA appears to be a significant step. Although, it was partially thought that IG was hydrolysed by salivary enzymes and stomach juice (Figure 2.3), there are still some disagreements (Allred et al., 2001; Kelly et al., 1993). Therefore, many studies have been conducted to break IG to liberate IA. Also, providing food products with IA would be considered a novel trend for the food industry.

2.3.1 The stability of soy isoflavones

Soy isoflavones are reported to be stable under several conditions including low and high temperature, acidic and basic conditions. Table 2.3 summarises the stability of SI under different conditions. In general, IG are stable in high temperature conditions. The malonyl conjugates were reported to be converted to either β-glycosides or acetyl conjugates but not aglycone forms. Similar results were reported in low acidic conditions. Furthermore, the malonyl and acetyl moieties provide more stability to isoflavone compounds, especially under acidic conditions (Mathias et al., 2006). However, in high pH condition, acetyl conjugates decreased while malonyl conjugates remained stable. No study reported that IA was released in high pH condition.

Table 2.3 Stability of SI under different processing conditions

Conditions	Stability	Reference
High temperature conditions		
Baking in an oven at 190 ^{o}C for 0–30 min.	Stable in general except malonyl conjugates partly converted to β-glycoside or acetyl conjugates,	(Coward et al., 1998)
Autoclaving at 120 ^{o}C for 15 min Boiling at 100 ^{o}C for 30 min	No significant change	(Setchell, 1998)
pH 7, at 100 ^{o}C for 2 hours	Malonyl daidzin partly transformed to daidzin	(Mathias et al., 2006)
Low temperature conditions		
Storage at -80 ^{o}C for 6 w	No significant change	(Otieno, Ashton, & Shah, 2006b)
Storage at -4 ^{o}C for 6 w	No significant change except genistin	(Otieno et al., 2006b)
Low pH conditions		
pH 2, at 37 ^{o}C, for 2 h	No significant change	(Ismail & Hayes, 2005)
pH 2, at 25 ^{o}C, for 2 h	No significant change for malonyl daidzin, acetyl daidzin, malonyl genistin and acetyl genistin	(Mathias et al., 2006)
High pH conditions		
pH 10, 25 ^{o}C, for 2 h	Acetyl genistin and acetyl daidzin decreased significantly Malonyl genistin, malonyl daidzin was stable	(Mathias et al., 2006)

2.3.2 Chemical hydrolysis of IG

Figure 2.7 show how base and acid hydrolyse IG molecule. To hydrolyse IG to IA, alkaline is utilised first. Basic hydrolysis breaks ester bonds, removing malonyl or acetyl moiety of the isoflavone glycosides. Then, acidic hydrolysis breaks the β-glucosidic bond between IA and their β-glycoside moieties (Figure 2.7). In the study of Delmonte et al. (2006), to hydrolyse IG to IA, the concentration of base (NaOH) and acid (HCl) was 0.14 and 2.7 M, respectively. The acid hydrolysis was carried out for 120 min at 80 ^{o}C. In the study of Utkina et al. (2004), IG were hydrolysed by HCl (6M) for 5 h at 100 °C resulting in a IA mixture and charred sugar. However, in these studies, the hydrolysis level of IG to IA was not mentioned clearly. In the basic condition (pH 10) and high temperature (100 °C), decarboxylation of malonyl daidzin into acetyl daidzin was also observed but at a very low level (3.3%) (Mathias et al., 2006). In general, the malonyl and acetyl moieties provide more stability to the isoflavone, especially under acidic conditions (Mathias et al., 2006).

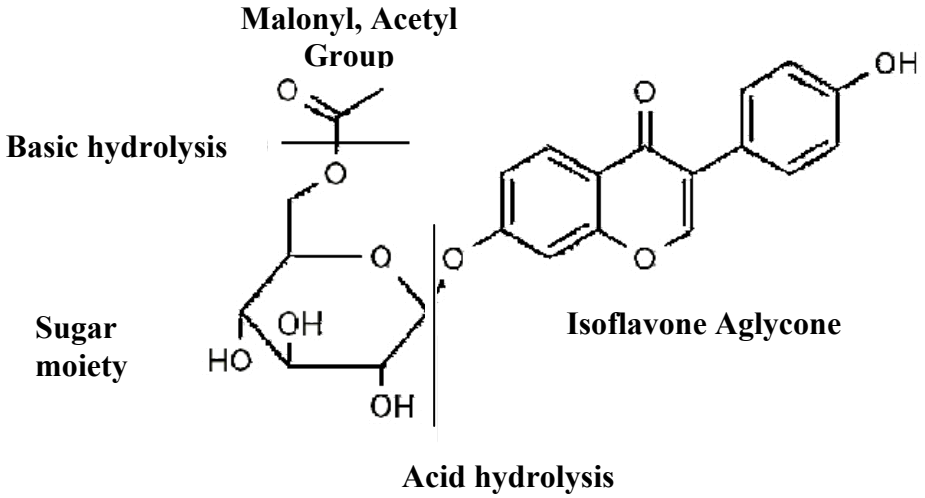


Figure 2.7 Basic and acidic hydrolysis of IG

Adapted from Delmonte, Perry, & Rader (2006)

2.3.3 Microbial transformation of IG to IA

2.3.3.1 Microorganisms used for microbial transformation of IG to IA

Since the gut microflora play a key role in the metabolism of SI, several groups have studied the biotransformation of IG to IA by microorganisms, especially those isolated from faeces (Chun et al., 2007). Lactic acid bacteria (LAB) and probiotic organisms have been also used widely. These bacteria produce lactic acid as the major metabolic end-product of carbohydrate fermentation. The genera that comprise the LAB are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus* as well as *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Teragenococcus*, *Vagococcus*, and *Weisella* (Holzapfel & Wood, 1998). Most of them have been employed to biotransform IG to IA, individually or in the mix culture. Apart from LAB, probiotic organisms such as bifidobacteria have been also used widely for the conversion. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Therefore, using LAB or probiotic organisms to ferment IG to IA in soy food, the final products such as fermented soymilk would have both health benefits from IA and the microorganisms. Table 2.4 summarises the microorganisms that have been used for the biotransformation of

IG to IA. As shown in Table 2.4, the most popular microorganisms that have been used for the transformation of IG to IA are *L. acidophilus, B. animalis, L. casei, L. delbrueckii* subsp. *bulgaricus, B. longum* and *S. thermophilus*. They are all considered to have high ability to produce β-glucosidase (Chien et al., 2006; Otieno et al., 2006a; Tsangalis et al., 2002). However, most microorganisms in as shown Table 2.4 can ferment lactose and produce lactic acid as the main end product of the metabolism. Therefore, they are also able to generate lactase, (β-galactosidase; EC. 3.2.1.23), as well.

Table 2.4 Microorganisms used for the biotransformation of IG to IA

Microorganisms	References
L. acidophilus	(Shelef, Bahnmiller, Zemel, & Monte, 1988; Chien et al., 2006; Otieno et al., 2006a; Farnworth et al., 2007; Wei, Chen & Chen, 2007; Donkor et al., 2007)
B. animalis	(Tsangalis et al. 2002; Otieno et al. 2006a; Farnworth et al., 2007; Donkor et al., 2007)
L. casei	(Choi, Sim & Rhee, 2002; Otieno et al., 2006a; Donkor et al., 2007)
L. delbrueckii subsp. *bulgaricus*	(Choi et al., 2002; Farnworth et al., 2007; Shelef et al.,1988)
B. longum	(Chien et al., 2006; Marotti et al., 2007; Tsangalis et al. 2002; Wei et al., 2007)
S. thermophilus	(Chien et al., 2006; Farnworth et al., 2007; Shelef et al., 1988)
B. catenulatum	(Marotti et al., 2007)
B. pseudocatenulatum	(Marotti et al., 2007)
B. adolescentis	(Marotti et al., 2007)
B. bifidum	(Marotti et al., 2007)
B. infantis	(Chien et al., 2006; Marotti et al., 2007)
B. breve	(Marotti et al., 2007)
B. pseudolongum	(Tsangalis et al., 2002)
S. infantarius	(Chun, Kim, & Kim, 2008)
S. salivarius	(Chun et al., 2007)
L. paracasei	(Wei et al., 2007)
L. paraplantarum	(Chun et al., 2007)
L. johnsonii	(Farnworth et al., 2007)
L. rhamnosus	(Farnworth et al., 2007)
Weissella confusa	(Chun et al., 2007; Chun et al., 2008)
Entcrococcus durans	(Chun et al., 2007)
Aspergillus oryzae	(Horiia et al., 2009)

The study of Tsangalis et al. (2002) reported that *B. longum* and *B. pseudolongum* could metabolise IG to equol, a final metabolite of daidzein in fermented soymilk.

2.3.3.2 The biotransformation level of IG to IA by microorganisms

The biotransformation level of IG to IA varies. Table 2.5 shows some examples of the biotransformation levels of IG to IA by microorganisms. As shown in Table 2.5, the biotransformation level varied widely ranging from 5.3 to 100%. The maximum level of

the biotransformation of IG to IA was 100% by *L. delbrueckii* subsp. *delbrueckii* KCTC 1047 in the study of Choi et al. (2002) after 12 h of incubation. However, the medium in this study was supplemented with glucose (2%) and the IG was genistin only (Table 2.5).

Table 2.5 Biotransformation levels of IG to IA by microorganisms

Microorganism	Transformation level (%)	Fermentation conditions	Reference
L. lactis KCTC 2181	75.0*	Fermented soymilk (supplemented with 2% glucose) at 12 h at 37 °C. IG was genistin only	(Choi et al., 2002)
L. delbrueckii subsp. *delbrueckii* KCTC 1047	100.0*	Fermented soymilk (supplemented with 2% (w/v) glucose) at 12 h at 37 °C. IG was genistin only	(Choi et al., 2002)
B. longum BCRC 14661	85.4*	Fermented soymilk (supplemented with 10% (w/v) of sucrose, fructose and lactose) at 24 h at 37 °C.	(Wei et al., 2007)
L. acidophilus	5.3*	Fermented soymilk at 32 h at 37 °C	(Chien et al., 2006)
B. longum	6.4*	Fermented soymilk at 32 h at 37 °C	(Chien et al., 2006)
L. acidophilus and B. infantis	10.7*	Fermented soymilk at 32 h at 37 °C	(Chien et al., 2006)

(*) Authors recalculated from to the data in the cited reference based on the formula:

$$\frac{\text{initial IG} - \text{residual IG}}{\text{initial IG}} \times 100$$

Generally, during the fermentation period, bacteria generate enzymes such as β-glucosidase and β-galactosidase to "digest" nutrients in the medium. These enzymes also hydrolyse IG to IA. When IG are broken to IA, the total amount of SI must decrease as β – glycosides generally comprise of nearly a half molecular weight of IG (as presented in Table 2.1). However, this is in contradiction with some studies, in which the total isoflavone compounds including IG and IA was reported to be constant during the entire period of fermentation although the level of biotransformation reached up to 61% by *S. thermophilus* and *B. longum* after 24 h at 37 °C (Chien et al., 2006).

2.4 Health benefits and side effects of soy isoflavones

Soybeans, especially SI may become the "ultimate women's health supplement of 21st century" (Challem, 1997). The incidence of cancers, such as breast and prostate, has been found to be much higher in Western populations compared with those in countries such as

Japan and China. In fact, Asian women who consume traditional high-soy diets have a relatively low incidence of reproductive and hormone-related disorders, including menopausal hot flashes, osteoporosis, and breast cancer. There are still some contradicting studies regarding health benefits and side effects of SI.

2.4.1 Relief of the menopausal symptoms

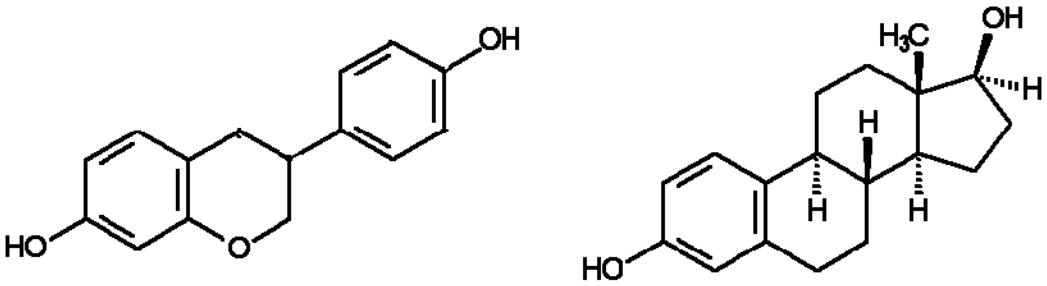
2.4.1.1 Menopausal symptoms

Menopause is the transition period in a woman's life when her ovaries stop producing eggs. Their bodies produce less estrogen and progesterone, and menstruation becomes less frequent, eventually it stops altogether. The symptoms of menopause are caused by changes in estrogen and progesterone levels. As the ovaries become less functional, they produce less of these hormones and the body responds accordingly (Medline Plus, 2008). The most common symptoms include heart pounding or racing, hot flashes, night sweats, skin flushes, and sleeping problems (insomnia). In the Western world, about 88% of women experience the symptoms of menopause and about 14% of them experience intense physical or emotional problems (Higdon, 2006). However, only 18% of Chinese and 14% of Singaporean women experience hot flushes (Knight, Wall, & Eden, 1996).

2.4.1.2 How soy isoflavone relieves menopausal symptoms without promoting breast cancer?

Soy isoflavones can bind to estrogen receptors, mimicking the effects of estrogen in some tissues due to the similarity of structural homology to female hormone. Figure 2.9 shows the chemical structure of equol and a female hormone, estradiol (17β-estradiol). Although the chemical structures of SI are similar to estradiol (Figure 2.9), they do not act exactly like estradiol. For this reason, SI possibly do not enhance the breast cancer risk like the hormone replacement therapy does to menopausal women (Women's Health Initiative, 2004). Estrogens have to bind to a 'receptor' to carry out its effects in the body. There are two main types of oestrogen receptors in the body, oestrogen receptor alpha (ERα) and oestrogen receptor β (ERβ). The reason why SI do not act the same way as estrogen is that they have a preference to bind to ERβ rather than ERα (Setchell & Cassidy, 1999). Hence, isoflavones have been suggested to be specifically named as nature’s selective estrogen receptor modulators (SERMs) (Brzezinki & Debi, 1999). Moreover, isoflavones bind

differently to estrogen receptors compared to estrogen, thus sending different signals. For example, genistein binds to estrogen receptors in a different way to estradiol, and more similarly to the way the anti-estrogen breast cancer drug raloxifene binds (Pike et al., 1999). A study of 7,700 postmenopausal women found that raloxifene was highly protective against breast cancer (Cummings et al., 1999; Sanitarium Health Food Company, 2004).



Equol

Estradiol (17β-estradiol)

Figure 2.8 Chemical structures of equol and estradiol

Adapted from Setchell & Cassidy (1999)

2.4.1.3 The effects of soy-enriched diets on menopausal women

The relieving effects of SI on the menopausal symptoms are summarised in Table 2.7. The studies which showed that SI had insignificant effects on menopausal symptoms were carried out for a very short term (4 and 12 weeks) even the diet was enriched with the high amount of SI (90-165 mg SI/day) (Baird et al., 1995; Van-Patten et al., 2002). For longer trial period, although the SI intake was low (35mg SI/day), the significantly improved effects on the menopausal symptoms (hot flushes) were still observed (Albert et al., 2002). Hence, SI may have effects of relieving menopausal symptoms if the subject consumes soy food or SI for at least in a period of 16 w as the results shown in the study of Jeri (2002). However, further studies are needed to verify if SI are able to alleviate the menopausal symptoms or an appropriate dose of SI is required to relieve the menopausal symptoms.

Table 2.6 Influence of isoflavone-enriched diet relieving menopausal symptoms

Studied Design	Diet with SI	Effects	References
		Significantly positive effects	
Japanese women (n =1106) aged between 35-54 years	44-115 g of soy product/d for 6 y	Consumption of soy products has a protective effect against hot flashes.	(Nagata, Takatsuka, Kawakami, & Shimizu, 2001)
Spanish postmenopausal women (n =190)	35 mg SI/day for 4 m	Hot flashes were decreased in 81% of participants	(Albert et al., 2002)
Double-blind, parallel, multicenter, randomized placebo-controlled trial (n = 104)	40 g of SPI /d For 12 w	SPI added daily to the diet reduced the frequency of hot flushes in climacteric women.	(Albertazzi et al., 1998)
Double-blind placebo controlled study (n = 40)	33.3 mg/d, for 4 m	significantly decreased menopausal symptoms ($p< 0.01$)	(Han et al., 2002)
Placebo-controlled double blind (n = 30)	40 mg of isoflavone/day for 16 w	48% reduction ($p<0.001$) in hot flushes	(Jeri, 2002)
		Insignificant effects	
Randomised, placebo-controlled trial (n = 97)	165 mg of isoflavones/day for 4 w	Non-significant increase in the percentage of vaginal superficial cells	(Baird et al., 1995)
Postmenopausal women (n=59) Control (n = 64)	90 mg of isoflavones/day for 12 w	No differences or insignificant in menopausal symptoms were reported	(Van-Patten et al., 2002)

2.4.2 Soy isoflavones and cancers

The effects of SI on cancers are still controversial. Allred et al. (2001) found that genitein and genistin stimulated growth of estrogen-dependent breast cancer tumours (MCF-7) and removal of genistin or genistein from diet caused tumours to regress into athymic mice. On contrast, it has been reported that genistein, behaves as a general cell growth inhibitor (Barnes, 1995). On contrast, genistein was reported to inhibit the growth of both estrogen receptor negative and positive breast cancer cells, such as MDA-MB-231, MDA-MB-435, and MCF-7 cells, PC3 and LNCaP prostate cancer cells (Li et al., 1999). Sarkar & Li (2003) reported that genistein inhibited the growth of cancer cells by modulating the expression of genes that are involved in the regulation of cell cycle and cell growth. The inactivation of Akt, NF-kB, p21, erb B-2/MMPs, and Bax/Bcl-2 signaling pathways may, thus, represent the molecular mechanism(s) by genistein exerts its anticancer effects (Sarkar & Li, 2003). The effects of soy/SI diet *in vivo* or *in vitro* experiment on cancer prevention are summarised in Table 2.8.

Table 2.7 Influence of isoflavone-enriched diet on cancer prevention

Studied Design	Diet with SI	Effects	References
		Significantly positive effects	
Asian-Americans (n = 597)	High tofu consuming >120 time/year Low tofu consuming <13 time/year	Lowered risk of breast cancer	(Wu et al., 1996)
Group 1: patients with newly diagnosed Group 2: patients with increasing serum (prostate-specific antigen) PSA Group 3: patients receiving hormone therapy.	100 mg SI/day for up to 6 mo	Induces apoptosis and inhibits growth of both androgen-sensitive and androgen-independent prostate cancer cells	(Hussain et al., 2003)
254 ovarian cancer patients	9 soy foods consumed up to 2 times/d	Higher intake of SI can protect against ovarian cancer.	(Zhang et al., 2004)
In vitro experiment on cell line of breast/prostate cancer		Metabolite of isoflavone, 2-de-O-DMA is inhibitor of hormonal cancer proliferation.	(Xiang et al., 2002)
In vitro experiment on cell line of prostate cancer		Genistein induces apoptosis in prostate cancer cells: NF-κB.	(Davis, Kucuk, & Sarkar, 1999)
Breast cancer patients (n = 362)	7.4 ± 4.5 g soy protein/d 27 ± 38 g tofu/d	Diet high in tofu and total soy protein intake may be strongly associated with a reduced risk of breast cancer	(Kim et al., 2008)
		Insignificant effects	
17 breast cancer patients	30–50 mg of SI/d for 2 w	Non significant cancer growth inhibition	(Sartippour et al., 2004)
58 men at high/low risk of prostate cancer	SPI consumed everyday Samples were taken at 0,3 and 6 mo	SPI had no effects on any of the prostate cancer tumour markers analysed.	(Hamilton-Reeves et al., 2008)
1,294 prostate cancer patients	24.9 μg SI/d	No significant beneficially effects	(Bosetti et al., 2006)

To confirm the effect of SI on the cancer prevention, a larger and longer term trial with higher SI intake should be carried out. In the studies of Sartippor et al. (2004), for example, only 17 breast cancer patients were examined and in the study of Bosetti et al. (2006), prostate cancer patients consumed only 24.9 μg of SI /day. Furthermore, soy-based foods are also claimed to have protective effect on lung cancer and stomach cancer (Nagata, 2000; Swanson et al., 1992). However, the results are not consistent and need further investigation.

2.4.3 Soy isoflavones and bone health

Soy isoflavones are reported to help in the preservation of the bones and fight osteoporosis. The effects of soy or isoflavone-enriched diet on osteoporosis prevention are summarised in Table 2.9.

Table 2.8 Influence of isoflavone-enriched diet on bone health

Studied design	Diet with SI	Effects	References
		Significantly positive effects	
Double-blind, placebo-controlled, randomized trial (n = 200 woman, aged: 48 - 62)	Group 1: placebo Group2: 40 mg SI/d Group 3: 80 mg SI/d for 1 year	SI have a mild, but significant effect on the maintenance of hip bone mineral density in postmenopausal women with low initial bone mass	(Chen et al., 2003)
Double-blind, placebo-postmenopausal women with a history of breast cancer (n = 55)	114 mg isoflavone/d for 3 months	Isoflavonoid induced inhibition of bone resorption	(Nikander et al., 2004)
Single-blind randomized, placebo-controlled trial (n = 90)	Placebo group: no SI Low-dose 84 mg SI/d High-dose: 126 mg SI/d For 6 mo	SI had a significantly positive dose-dependent effect on attenuating bone loss at the spine and femur neck possibly via the inhibition of bone resorption in postmenopausal Chinese women.	(Ye et al., 2006)
Japanese women (n = 944)	Natto consumption Group 1:1-4 times/w Group 2: > 4 time/w	Natto intake may decrease the loss of bone mass at the femoral neck	(Ikeda et al., 2006)
Female mice	Equol intake 0.1 mg/d or 0.5 mg/d For 4 w	Equol inhibits bone loss without estrogenic activity in the reproductive organs of mice	(Fujioka et al., 2004)
		Insignificant effects	
Postmenopausal < 5 years (n = 269) Postmenopausal >5 years (n = 209)	High soy food consumption (~54.3 mg SI / day)	Insignificant effects on menopausal symptoms were reported	(Somekawa et al., , 2001)

Among SI compounds, daidzein was reported to be more efficient than genistein in preventing ovariectomy-induced bone loss in rats (Picherit et al., 2000). Normally, studies were conducted for a short duration and involved a small number of subjects. Although the data in general are encouraging for consumption of SI, no firm conclusions have been drawn. The mechanisms of action of SI on bone health are not fully investigated. Therefore further studies regarding the influence of SI- enriched diet on the bone health are required to have confirmative conclusions and the relationship between the SI and their metabolites and bone health is still unclear (Weaver & Cheong, 2005).

2.4.4 Soy isoflavones and cardiovascular system

Soy isoflavones are reported to have effects on cardiovascular system as well. In fact, in 1999, the Food and Drug Administration authorized a health claim for the cholesterol-lowering potential of modest intakes of soy protein. However, this has been controversial partly as some studies showed that SI had no significant effect on cardiovascular system (Nestel, 2002). Table 2.10 summarises the effects of SI on cardiovascular system such as high density lipoprotein cholesterol (HDLC), low density lipoprotein cholesterol (LDL), and total plasma cholesterol (TPC).

Table 2.9 Influence of isoflavone-enriched diet on cardiovascular system

Studied Design	Diet with SI	Effects	References
		Significantly positive effects	
Rhesus monkeys (n=27)	SPI: 200 g/kg diet For 6 mo	Significantly reducing (~30-40%) LDLC+ VLDL, increasing HDLC 15%, lowering TPC	(Anthony et al.,1996)
Male monkeys	Group 1: (n =30), non SI Group 2: (n = 30): 0.94 mg of SI /g protein; Group 2: (n = 31): 1.88mg of SI /g protein for 31 mo	Long-term consumption of SPI containing of isoflavones inhibits the early progression of coronary artery atherosclerosis without affecting endothelium-dependent or -independent arterial function	(Adams et al., 2005)
Ovariectomized monkeys (n=17-20 each group)	Group 1: casein/lactalbumin Group 2: SPI Group 3: casein/lactalbumin with SI	Group 2: Coronary artery LDL degradation was reduced by 50%	(Wagner et al., 2003)
		Insignificant effects	
A placebo-controlled trial, (n=21)	45 mg genistein/d 5- 10-week	Plasma lipids were not changed	(Nestel et al., 1997)
Male cynomolgus monkeys (n = 27-28 each group)	Group 1: casein/lactalbumin Group 2: SPI with 0.17 mg SI/g SPI Group 3: SPI with 1.5 mg SI/g SPI for 14 mo	No significant effects between group 2 and 3	(Anthony et al.,1997)

HDLC: high density lipoprotein cholesterol; LDL: low density lipoprotein cholesterol; TPC: total plasma cholesterol,

Lichtenstein (1998) proposed the following potential mechanisms for beneficial effects of soy protein on decreasing cardiovascular disease risk:

- Decrease in the plasma cholesterol levels

- Increase in bile acid excretion
- Increase in LDL receptor activity
- Decrease in cholesterol absorption
- Decrease in susceptibility of LDL to oxidation
- Increase in arterial compliance
- Estrogenic activity of soy isoflavones

2.5 Possible side effects of soy food and SI

These are several side effects of SI that have been reported. Table 2.11 summarises the possible side effects and concerns of consuming SI.

Table 2.10 Possible side effects/concerns of consuming SI

Possible effects/concern	Solutions/Response	References
Alteration of immune system		
Soy isoflavones affect immune system.	Studies showed that SI enhance immune function in healthy postmenopausal women. SI prevent the ovarian hormone deficiency-associated rise in leukocytes in rats.	(Ryan-Borchers et al., 2006; Sounga et al., 2004)
Goiter		
SI are goitrogenic. Soy foods contain goitrogens that can cause an enlarged thyroid (goiter).	Soy and SI could cause goiter, but only in those consuming diets marginally adequate in iodine, or who were predisposed to develop goiter.	(Divi, Chang & Doerge, 1997; Doerge & Sheehan, 2002; Messina & Messina, 2003)
Dementia /Cognition		
Higher midlife tofu consumption was independently associated with indicators of cognitive impairment and brain atrophy in late life.	Still remains controversial. Other studies suggest eating soy product improve memory.	(File et al., 2001; White et al., 2000)
Affecting reproductive functions		
Soy food/SI affect male reproductive function parameters (e.g.: reproductive hormones and semen quality).	The consumption of 40 mg of SI/d had no effect. SI do not lower the sperm count.	(Kurzer, 2002; Mitchell et al., 2001)
Soy consumption delays ovulation.	Still not clear. SI do not prevent ovulation	(Kurzer, 2002
Poorer mineral absorption		
Soy foods are high in phytates which inhibit absorption of iron, zinc, and calcium	The isoflavones in soy foods improve bone health as shown in several studies (Table 2.9).	(Chen et al., 2003; Nikander et al., 2004; Ye et al., 2006)

2.6 Summary of literature review

Overall, most scientists, who studied SI and the influence of SI on health, agreed that:

- IA are more bioavailable than IG. It is necessary to biotransform IG to IA since the biotransformation level varies with individual and as IG are the dominant isoflavone group in soy foods (especially non-fermented soy food).
- The metabolism of SI in human body is not fully clear (especially about the final products of the metabolism). The gut microflora play an essential role in the metabolism of SI. Therefore, there are several factors that affect the metabolising rate such as gender, diet and medication.
- Apart from chemical hydrolysis to transform IG to IA, microbial hydrolysis has become popular where both β-glucosidase and β-galactosidase break the β-glucosidic bond between IG and IA.
- Recently, several methods have been introduced to enhance the biotransformation level of IG to IA. For example, by adding lactulose, a prebiotic, the biotransformation of IG to IA increased up to 20.6% by probiotic organisms.
- The health benefit of SI on menopausal symptoms, bone health, cardiovascular system and prevention of cancer is relatively clear. However, there are several studies that reported insignificant effects or even negative effects on human health. Yet, no firm conclusion has been drawn as all studies suggested that consuming soy food and SI might be the reasons. Nevertheless, the health benefits of SI triumph over the side effects. Comparing the number of side effects reports, the beneficial effect studies are greater in number. Since the metabolism of SI strongly depends on each individual such as their life style, gender and gut microflora, SI might provide significant health benefits to one individual but not notably to others. Even so, the health effects of SI deserve to be studied more thoroughly, in larger and longer-term clinical trials with variable concentration of SI in different locations over the world.

Chapter 3.0

Hydrolysis of isoflavone glycosides in soymilk by β-galactosidase and β-glucosidase

This chapter has been published:

Pham, T. T**.**, & Shah, N. P. (2009). Hydrolysis of isoflavone glycosides in soymilk by β-galactosidase and β-glucosidase. **Journal of Food Biochemistry**, 33, 38-60

3.1 Introduction

Isoflavones, the main flavonoid class of phytoestrogens, are mostly found in legumes such as soybeans. In nature, they exist in 2 forms, aglycones, the non-sugar moiety and aglycones conjugated with β-glycosides. The chemical structures of these isoflavones are shown in Figure 2.2. However, only the biologically active forms, aglycones, have been reported to have an estrogenic effect (Hughes et al., 2003; Setchell, 2001; Setchell et al., 2001; Setchell & Cassidy, 1999). As the chemical structures of aglycones are estrogen-like, they are able to bind to estrogen receptor sites and therefore mimic the function of estradiol and relieve the menopausal symptoms (Setchell & Cassidy, 1999; Tsangalis et al., 2002). There are 5 aglycone compounds in isoflavonoid group including daidzein, glycitein, genistein, biochanin A and formononetin. The biologically active forms comprise a minor percentage (approximately 3-5%) of isoflavone compounds in nature as well as in non- fermented soy food (Hughes et al., 2003; King & Bignell, 2000; Nakamura et al., 2001). Although isoflavone glycosides (IG) are partly hydrolysed to aglycones by saliva then by the gut microflora, the rate of the transformation is low (Allred et al., 2001; Xu et al., 1995). In addition, the rate of transformation also varies with individuals. Only 33% of individuals in Western populations are able to metabolise in the intestinal tract daidzein glucosides to daidzein and daidzein to equol, the metabolite which has stronger estrogenic effect (Frankenfeld et al., 2005; Higdon, 2006). Therefore, it is beneficial to transform IG to biologically active forms before consumption. Consequently, providing food products enriched with aglycones as the main components of isoflavones would be considered as a novel trend for the food industry.

To deconjugate IG to biologically active aglycones, the β-glucosidic linkage between the β-glycoside and aglycones must be broken. In the last few years, several scientists have reported on the transformation of IG to aglycones by chemical hydrolysis and microbial fermentation. Transformation of IG to aglycones can be achieved using a base and an acid. Ester bond in the acetyl- and malonyl- group of β-glycoside part is hydrolysed using a base. An acid can be applied to hydrolyse the bonds between the aglycones and the glycoside moieties. These chemical processes are normally used for food analysis. Tsangalis et al. (2002) studied the enzymic transformation of isoflavone phytoestrogens in

soymilk by β-glucosidase-producing *Bifidobacterium*. Otieno et al. (2006a) reported the evaluation of enzymic potential for biotransformation of isoflavone phytoestrogen in soymilk by *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus casei*. Similary, Chien et al. (2006) studied the transformation of isoflavones during the fermentation of soymilk with lactic acid bacteria and bifidobacteria. In these studies, β-glucosidase (EC 3.2.1.21) produced by bacteria was claimed to be responsible for the biotransformation of IG to aglycones. However, bacteria such as *Bifidobacterium* produce β-galactosidase (EC 3.2.1.23) in addition to β-glucosidase (Shah, 2006; Shah & Jelen, 1990). Regarding the specificity, β-galactosidase is classed as the linkage specific enzyme. The enzyme acts on a particular type of chemical bond, which is β-galactosidic bond, regardless of the rest of the molecular structure. β-galactosidase was reported not to be strictly specific to the β-galactosidic bond. It was shown that β-galactosidase could also hydrolyse the α-galactosidic bond in α-lactose (Huber, Hurlburt, & Turner, 1981). Therefore, it is possible that β-galactosidase is also responsible for the biotransformation of IG to aglycones since the β-galactosidic bond and β-glucosidic bond are relatively similar. If β- galactosidase can be proven to hydrolyse IG to aglycones, a novel and more resourceful method to produce aglycones will be discovered, as there are more sources of β-galactosidase and certain lactic acid bacteria produce this enzyme in abundance (Shah, 1993). To date, no study has reported the biotransformation of IG to biologically active forms by β-galactosidase. Therefore, the objectives of this work were to assess whether pure β-galactosidase could be able to hydrolyse IG and to examine the effectiveness of pure β-glucosidase on the biotransformation of IG to aglycones in soymilk.

3.2 Materials and Methods

3.2.1 Isoflavones and other chemicals

Genistein, daidzein, glycitein and flavone were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Daidzin, glycitin, genistin, formononetin, and biochanin A were obtained form Indofine Chemical Company, Inc. (Somerville, NJ, USA). Malonyl- and acetyl- β glycosides (malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin) were obtained from LC Labs (Woburn, MA, USA). Acetonitrile, methanol and phosphoric acid for high performance liquid chromatography

(HPLC) were of analytical grade. The water used was deionised milli-Q grade. β-glucosidase and β-galactosidase were from Sigma-Aldrich with more than 98% of the purity.

3.2.2 Preparation of soymilk

Soy protein isolate (SPI) SUPRO 590 was supplied from The Solae Co. (Chatswood, NSW, Australia). Five litres of soymilk were prepared using 4% (w/v) SPI. A 100 mL of the soymilk aliquot was freeze-dried for HPLC analysis. The rest of the soymilk solution was autoclaved at 121 ^{o}C for 15 min in order to inactivate endogenous enzymes which may affect the hydrolysis of IG.

3.2.3 Hydrolysis of p-nitrophenyl-β-D glucopyranoside (p-NPG) by β-galactosidase and β-glucosidase

A 2.0 U/mL solution of β-galactosidase (98%) and β-glucosidase (98%) were prepared separately in sodium phosphate buffer (0.1 M, pH 7.0). p-NPG was prepared at 5mM in sodium phosphate buffer (0.1M, pH 7) then covered to avoid light. Nine millilitres aliquot of p-NPG solution were incubated with 1 mL of β-galactosidase or β-glucosidase separately at 37 ^{o}C for 30 min. The amount of p-nitrophenol released was measured at 420 nm using a spectrophotometer (Pharmacia LKB, Novospec II, Uppsala, Sweden). One unit of enzyme activity was defined as the amount of enzyme that released 1 μM of p-nitrophenol from the p-NPG substrate per millitre per minute under assay conditions as described by Tsangalis et al. (2002).

3.2.4 Hydrolysis of soymilk by β-galactosidase and β-glucosidase

The autoclaved soymilk made from (4%, w/v) was adjusted to pH 6.5 from an initial pH 6.8 for optimum β-galactosidase activity and to pH 5.0 for β-glucosidase using 5M hydrochloric acid. β-galactosidase and β-glucosidase were added separately at various concentrations (0.5 U/mL, 1.0 U/mL, 2.0 U/mL and 4.0 U/mL) and incubated at 37 ^{o}C for 30, 60, 120, 180 and 240 min in triplicate. Samples were withdrawn after each incubation period and freeze-dried using a Dynavac freeze-dryer (model FD300; Rowville, VIC, Australia) for the determination of isoflavones.

3.2.5 Extraction of isoflavones

Extraction of isoflavone was performed in triplicate according to Griffith & Collison (2001) with some modifications. Briefly, one gram of freeze-dried sample was mixed thoroughly with 20 mL of methanol (80%) and 100 µL of the internal standard flavone (1 mg/mL) into a 50 mL screw cap tube. Isoflavones were extracted at 50 ^{o}C for 120 min in a water bath (model NB 6T-10935; Thermoline Australia Scientific Equipment, Smithfield, NSW, Australia). After thorough shaking, the aliquots were then filtered through a Whatman No. 3 filter paper. One millilitre of the filtered solution was passed through a 0.45 µm Phenomenex nylon filter (Lane Cove, NSW, Australia) into a HPLC vial then injected into HPLC system within 4 hours to avoid the degradation of malonyl- and acetyl –glycosides (Griffith & Collison, 2001).

3.2.6 HPLC method

The HPLC method was based on Nakamura et al. (2001) with some modifications.

Instrument: An Alltech Alltima (Deerfield, IL, USA) HP C18 HL (4.6 mm i.d. x 250 mm, 5 µm particle size) column and an Alltima HP C18 HL (7.5 mm x 4.6 mm internal diameter, 5 µm) guard column. A Hewlett Packard 1100 series HPLC (Agolient Technologies, Forest Hill, VIC, Australia) with an auto sampler, a quaternary pump, a diode array, a UV detector, a vacuum degasser and a thermostatically controlled column compartment. Column temperature was maintained at 25 ^{o}C. The injection volume was 20 µL.

Mobile phase: Solvent A (water: phosphoric acid, 1000:1, v/v) and solvent B was (water: acetonitrile: phosphoric acid, 200:800:1, v/v/v). pH of solvent A and B was approximately 2.5. All the reagents used in the mobile phase were filtered through a 0.45 µm membrane (Millipore, Bedford, MA, USA). The gradient was: Solvent A 100% (0 min) → 0% (50 min) →100% (60 min). The flow rate was 0.8 mL/min and the UV detector was set at 259 nm. Stock solutions for 14 isoflavones were prepared separately by dissolving 1 mg of the crystalline pure compound in 10 mL of 100% methanol. Each solution was diluted with 100% methanol to 5 working solutions at various concentrations (1 µg/mL - 40 µg/mL) in order to prepare standard curves. All the working standards were injected into HPLC

system within 4 hours after preparation. Retention time and UV absorption patterns of pure isoflavonoid standards were used to identify isoflavones. Then, isoflavone concentrations were calculated back to dry basis (mg/100 g of freeze-dried soymilk). The moisture content of the freeze-dried soymilk samples was determined by AACC 40-40 method as the moisture contents affected the isoflavone contents (American Association of Cereal Chemist, 2000)

Statistical analysis of data: The quantification of isoflavones was performed in triplicate. The data were analysed by using one-way analysis of variance (ANOVA) and 95% confidence levels using Microsoft Excel Statpro (Allbright et al., 1999)

3.3 Results and Discussion

3.3.1 HPLC analysis of isoflavones

The HPLC chromatogram and the retention time of 14 standard isoflavone compounds and the internal standard are shown in Figure 3.1. Normally, daidzein-glycosides and glycitein-glycosides are co-eluted as their chemical structures are similar. However, the co-elutions were resolved by the gradient method reported in this study. The order of elution of the isoflavones was dependent on the polarity and hydrophobic interaction with the HPLC column (Tsangalis et al., 2002). In the studies of Nakamura et al. (2001), biochanin A eluted after formononetin while our results showed a reverse order. Flavone, the internal standard, eluted at 42 min and segregated from isoflavone compounds to prevent overlapping. The detection limit of HPLC analysis was approximately 10^{-8} g/mL.

3.3.2 Comparison of isoflavone content of soymilk before and after autoclaving

The moisture content of SPI powder was 4.5 ± 0.1% and that of freeze-dried samples ranged from 1.9 -2.0%. There were no significant differences in moisture contents of the freeze-dried samples ($P > 0.05$). Therefore, it is assumed that there was no effect of the moisture content on the determination of isoflavones.

Isoflavone contents in soymilk before and after autoclaving are shown in Table 3.1 and Figure 3.2. As shown in Table 3.1, there was no significant difference ($P > 0.05$) in the

isoflavone contents in soymilk before and after autoclaving. These results are similar to those of Setchell (1998) who reported a slight reduction in daidzin, genistin, daidzein and genistein contents in soy flour and miso only reduced slightly after 30 min of boiling. Similarly, cooking was also reported to reduce phytoestrogen concentrations and convert malonyl-glucosides into acetyl-glucosides (Hughes et al., 2003; Wang & Murphy, 1994). In agreement, our results specifically confirmed the thermostable characteristic of 8 isoflavonoid compounds namely daidzin, malonyl daidzin, acetyl daidzin, glycitin, malonyl glycitin, malonyl genistin, acetyl genistin and genistein (Table 3.1).

As presented in Table 3.1, more than half of isoflavones in soymilk were malonyl-glycosides including malonyl daidzin (24.49 ± 1.69 mg/100 g) and malonyl genistin (67.23 ± 2.02 mg/100 g). The second largest group was acetyl glycosides which included acetyl genistin and acetyl daidzin at 27.5 ± 1.63 mg/100 g and 6.41± 0.19 mg/100 g, respectively. Genistein was the only aglycone detected in soymilk which was found at a very small concentration of 4.50 ± 0.32 mg/100 g, which was about 2.9% of the total isoflavone. However, according to King & Bignell (2000), the dominant isoflavone group in soy flour was β-glycosides (including daidzin, glycitin and genistin), comprising approximately 51% of the total isoflavone. In addition, genistin content (77.0 mg/100 g) was moderately high in their study while no genistin was detected in our study. Moreover, there were no biochanin A, formononetin, acetyl glycitin, daidzein and glycitein detected in soymilk before and after autoclaving. Similarly, no formononetin and biochanin A were detected in mature soy bean and soy bean sprouts in other studies (Klejdus et al., 2005; Nakamura et al., 2001). The decrease in level of the isoflavone compounds and the absence of some isoflavone compounds in soymilk may be due to losses during isolation of SPI. Wang & Murphy (1994) reported that mild alkali extraction used in the production of SPI caused isoflavones losses of 53%.

3.3.3 Hydrolysis of p-NPG by pure β-galactosidase and β-glucosidase

The effectiveness of pure β-galactosidase and β-glucosidase on the hydrolysis of p-NPG was assessed. It is hypothesized that if β-galactosidase was able to hydrolyse the β-glucosidic bond in p-NPG molecule, then the hydrolysis of IG to aglycones with β-galactosidase could be expected. The results showed that the β-galactosidase activity was

Table 3.1 Isoflavone contents in soymilk before and after autoclaving

Isoflavones	*Isoflavone contents of soymilk before autoclaving (mg/100 g of dry matter)*	*Isoflavone contents of soymilk after autoclaving (mg/100 g of dry matter)*
Daidzin	14.50 ± 0.41[a]	14.03 ± 0.70[a]
Glycitin	6.33 ± 0.18[a]	6.13 ± 0.10[a]
Genistin	ND	ND
Malonyl daidzin	24.80 ± 1.04[a]	24.49 ± 1.69[a]
Malonyl glycitin	3.13 ± 0.38[a]	3.02 ± 0.07[a]
Malonyl genistin	68.52 ± 1.31[a]	67.23 ± 2.02[a]
Acetyl daidzin	6.22 ± 0.33[a]	6.41 ± 0.19[a]
Acetyl glycitin	ND	ND
Acetyl genistin	27.01 ± 2.12[a]	27.50 ± 1.63[a]
Total of IG	**150.51 ± 0.87[a]**	**148.81 ± 2.88[a]**
Daidzein	ND	ND
Glycitein	ND	ND
Genistein	4.95 ± 0.63[a]	4.50 ± 0.32[a]
Biochanin A	ND	ND
Formononetin	ND	ND
Total of IA	**4.95 ± 0.63[a]**	**4.50 ± 0.32[a]**

Results expressed as mean ± standard error (n=3). Statistical analysis by means of one-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different (P>0.05). ND: Not detected (the isoflavone content which was in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 μL was lower than the detection limit of the method)

0.184 ± 0.053 U/mL while β-glucosidase activity was 0.168 ± 0.069 U/mL. β-Galactosidase was reported to act on α-lactose more than twice as rapidly as on β-lactose for both the hydrolysis and transgalactosylis reactions (Huber et al., 1981). Hence, β-galactosidase did not appear to be a very specific enzyme as it is able to act on several glycosidic linkages including β-galactosidic-, β-glucosidic-, and α- glucosidic bonds.

3.3.4 Hydrolysis of IG by β-galactosidase

The hydrolysis of isoflavones in soymilk using various concentrations of β-galactosidase is shown in Tables 3.2 to 3.5. As shown in Table 3.2, the hydrolysis of IG was achieved even at the lowest concentration of β-galactosidase (0.5 U/mL). At 240 min, 43.3% of the total of IG was deconjugated and approximately 32 mg of aglycones per 100 g of freeze-dried sample was produced. Malonyl genistin was hydrolysed the most (36 mg/100 g) while acetyl daidzin was hydrolysed the least (0.7 mg/100 g). Consequently, genistein was produced the most (28.02 ± 1.38 mg/100 g) within 240 min (Table 3.2).

Table 3.3 presents the hydrolysis of soymilk by β-galactosidase at 1.0 U/mL. Compared with the hydrolysis of isoflavones in soymilk at 0.5 U/mL, the IG hydrolysed and the aglycones produced were significantly different ($P < 0.05$). At 240 min, 41.13 mg of aglycones per 100 g was produced by hydrolysing 57.8% of the total of IG. Most of IG were hydrolysed at a higher rate at 1.0 U/mL than that by β-galactosidase at 0.5 U/mL, except acetyl genistin.

The hydrolysis of soymilk by β-galactosidase at 2.0 U/mL is presented in Table 3.4. At this concentration, the enzyme acted much more effectively than at 1.0 U/mL. Most of IG were de-conjugated approximately 50% within 120 min. At 240 min, 69.5% of the total IG was hydrolysed and the total aglycone content attained was 57.51 mg/100g. The enzymatic reaction occurred more rapidly by β-galactosidase at 4.0 U/mL (Table 3.5). More than 50% of total IG was de-conjugated within 60 min. At 120 min, 70.2 % of the total IG were hydrolysed (Table 3.5). In general, during the hydrolysis of isoflavones in soymilk by β-galactosidase, the IG reduced rapidly in the first 120 min and then slowly thereafter. Consequently, the aglycone amounts produced were fairly stable after 120 min of the enzymatic reaction. There was no significant difference ($P >0.05$) between the residual IG and the aglycones produced after 180 min and 240 min at all concentrations of β-galactosidase studied as the rate of the enzymatic reaction had previously reached the maximum (Tables 3.2 to 3.5).

3.3.5 Hydrolysis of IG by pure β-glucosidase

The hydrolysis of isoflavones in soymilk at various concentrations of β-glucosidase (0.5, 1.0 and 4.0 U/mL) is shown in Tables 3.6 to 3.8. Table 3.6 presents the hydrolysis of isoflavones in soymilk at the most diluted concentration of β-glucosidase (0.5 U/mL). After the first 30 min of enzymatic reaction, the total aglycones increased from 4.5 ± 0.32 to 62.8 ± 4.34 mg/100 g sample by hydrolysing 75.1% of the total IG. Daidzin, glycitin and acetyl daidzin were completely hydrolysed. At 240 min, 86.7% of the total IG was hydrolysed. All of the IG present in soymilk were completely hydrolysed except malonyl genistin and acetyl genistin (Table 3.6).

Table 3.7 shows the hydrolysis of soymilk by β-glucosidase at 1.0 U/mL. At 30 min, nearly 80% of IG were hydrolysed to aglycones, 80% of malonyl genistin was deconjugated while only 47% of malonyl glycitin was hydrolysed. In contrast, in the study of Tsangalis et al. (2002), during the fermentation of soymilk by *B. pseudolongum*, about 89% of malonyl glycitin was hydrolysed compared to 24% of malonyl genistin in the first 12 hours. At 240 min, 69.62 mg of aglycones were produced per 100 g of sample by hydrolysing 87.5% of total IG by β-glucosidase at 1.0 U/mL (Table 3.7). Similar to the hydrolysis of soymilk by β-glucosidase at 0.5 U/mL, there was no significant difference ($P > 0.05$) in the residual IG and the aglycones produced at 180 and 240 min (Tables 3.6 and 3.7). The result of the hydrolysis of isoflavones in soymilk by β-glucosidase at 2.0 U/mL is not shown since there was no significant difference ($P > 0.05$) compared with that at 1.0 U/mL.

Table 3.8 and Figure 3.3 show the hydrolysis of soymilk by the highest concentration of β-glucosidase at 4.0 U/mL. Compared to the hydrolysis of soymilk by β-glucosidase at 0.5 and 1.0 U/mL, the total residual isoflavone glycoside contents were significantly lower ($P < 0.05$) during the whole enzymatic reaction. The enzymatic reaction occurred rapidly as almost all IG were hydrolysed in the first 30 min. At 240 min, about 93% of the total IG was hydrolysed. Malonyl genistin and acetyl genistin were still present at 240 min, although at a very low concentration. Consequently, daidzein, glycitein and genistein were produced in the largest quantities at 24.20 ± 1.21, 3.82 ± 0.32 and 51.92 ± 2.54 mg/ 100 g of freeze-dried samples, respectively (Table 3.8).

In general, the enzymatic reactions occurred rapidly in the first 30 min at all concentrations of β-glucosidase, and consequently, the amount of IG decreased and aglycone contents increased rapidly. Thereafter, the reaction slowed down. Isoflavone glycosides were deconjugated almost completely at 240 min, except malonyl- and acetyl genistin.

It appears that β-glucosidase hydrolysed IG much more efficiently than β-galactosidase. At the same time and concentration, IG were always hydrolysed by β-glucosidase at significantly higher levels than that by β-galactosidase. Compared with β-galactosidase, β-

glucosidase is specific for β-glucosidic bond. As both the enzyme and the substrate possess specific complementary geometric shapes that fit exactly into one another, β-glucosidase acts on IG as "lock and key" model (Fischer, 1894).

Interestingly, during the hydrolysis by both enzymes, neither biochanin A nor formononetin detected. It is, therefore, concluded that both biochanin A-glycoside (ononin) and formononetin-glycoside (sissotrin) were not present at levels above the detection limit.

3.4 Conclusions

In this study, β-galactosidase was proven to hydrolyse IG in soymilk to their aglycones even at a very low concentration of 0.5 U/mL. This suggests the lack of specificity of β-galactosidase, therefore it is able to hydrolyse the β-glucosidic linkage in IG molecules. Although compared to β-glucosidase, β-galactosidase was less efficient, this finding could open a novel method to produce aglycones by β-galactosidase instead of β-glucosidase. Since the β-galactosidase producing organisms are abundant, the production of isoflavone aglycones would be more efficient.

Table 3.2 The hydrolysis of IG in soymilk by pure β-galactosidase (0.5 U/mL)

Isoflavones (mg/100 g of freeze-dried sample)	*0 min*	*30 min*	*60 min*	*120 min*	*180 min*	*240 min*
Daidzin	14.03 ± 0.70^{a}	14.04 ± 1.12^{a}	12.28 ± 0.76^{ab}	10.90± 0.89^{b}	8.21± 0.75^{c}	7.97± 0.56^{c}
Glycitin	6.13 ± 0.10^{a}	6.11± 0.52^{ab}	6.03 ± 0.56^{ab}	5.11± 0.23^{bc}	4.12± 0.32^{c}	4.00± 0.24^{c}
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	24.49 ± 1.69^{a}	21.85 ± 1.36^{ab}	20.65 ± 1.15^{ab}	19.36± 1.42^{b}	18.97 ± 1.21^{b}	18.81± 1.52^{b}
Malonyl glycitin	3.02 ± 0.07^{a}	2.63 ± 0.13^{b}	2.37 ± 0.10^{b}	1.95± 0.11^{c}	1.83 ± 0.11^{c}	1.23± 0.07^{d}
Malonyl genistin	67.23 ± 2.02^{a}	59.18 ± 4.32^{ab}	53.37 ± 3.22^{b}	34.01± 0.85^{c}	32.78 ± 2.14^{c}	31.60± 1.69^{c}
Acetyl daidzin	6.41 ± 0.19^{a}	6.06 ± 0.56^{a}	6.03 ± 0.45^{a}	6.03± 0.38^{a}	5.93 ± 0.35^{a}	5.76± 0.84^{a}
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	27.50 ± 1.63^{a}	20.96 ± 2.11^{b}	20.60 ± 1.23^{b}	18.47± 1.12^{bc}	15.79 ± 1.24^{c}	14.95± 0.98^{c}
Total of IG	**148.81 ± 2.88^{a}**	**130.83 ± 3.40^{b}**	**121.33 ± 6.35^{b}**	**95.83 ± 3.56^{c}**	**87.63 ± 2.78^{c}**	**84.32 ± 4.16^{c}**
Daidzein	ND	2.02 ± 0.13^{a}	3.55± 0.21^{b}	5.37± 0.54^{c}	6.30± 0.54^{cd}	6.98 ± 0.57^{d}
Glycitein	ND	ND	1.14± 0.05^{a}	1.22± 0.14^{a}	1.36± 0.12^{a}	1.42 ± 0.14^{a}
Genistein	4.50 ± 0.32^{a}	9.77 ± 0.85^{b}	14.48± 0.84^{c}	27.62± 1.65^{d}	28.01± 1.26^{d}	28.02 ± 1.38^{d}
Biochanin A	ND	ND	ND	ND	ND	ND
Formononetin	ND	ND	ND	ND	ND	ND
Total of aglycones	**4.50 ± 0.32^{a}**	**11.79 ± 0.98^{b}**	**19.17± 1.10^{c}**	**34.21± 0.97^{d}**	**35.67± 1.92^{d}**	**36.42 ± 2.09^{d}**

Results expressed as mean ± standard error (n=3). Statistical analysis by means of one-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different (P>0.05) ND: Not detected (the isoflavone content which was in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method)

Table 3.3 The hydrolysis of IG in soymilk by pure β-galactosidase (1.0 U/mL)

Isoflavones (mg/100 g of freeze-dried sample)	*0 min*	*30 min*	*60 min*	*120 min*	*180 min*	*240 min*
Daidzin	14.03 ± 0.70^{a}	10.97 ± 0.91^{b}	10.46 ± 0.65^{b}	9.93 ± 0.86^{b}	8.70 ± 0.96^{bc}	7.37 ± 0.53^{c}
Glycitin	6.13 ± 0.10^{a}	6.01± 0.63^{ab}	5.11± 0.32^{bc}	4.21± 0.24^{c}	3.00 ± 0.23^{d}	3.12 ± 0.13^{d}
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	24.49 ± 1.69^{a}	21.66 ± 1.32^{ab}	20.30 ± 1.22^{bc}	16.94 ± 0.93^{c}	15.14 ± 0.91^{c}	15.31 ± 0.98^{c}
Malonyl glycitin	3.02 ± 0.07^{a}	2.00 ± 0.11^{ab}	1.45 ± 1.01^{ab}	0.95 ± 0.14^{b}	0.96 ± 0.13^{b}	0.81 ± 0.77^{b}
Malonyl genistin	67.23 ± 2.02^{a}	40.06 ± 2.65^{b}	23.54 ± 2.11^{c}	18.03 ± 1.19^{cd}	16.49 ± 1.02^{d}	15.68 ± 1.01^{d}
Acetyl daidzin	6.41 ± 0.19^{a}	5.62 ± 0.45^{a}	5.53 ± 0.62^{a}	5.41± 0.75^{a}	5.28 ± 0.32^{a}	4.92 ± 0.29^{a}
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	27.50 ± 1.63^{a}	18.66 ± 1.17^{b}	16.54 ± 1.13^{b}	15.29 ± 0.89^{b}	15.84 ± 0.81^{b}	15.61 ± 0.75^{b}
Total of IG	**148.81 ± 2.88^{a}**	**104.98 ± 2.56^{b}**	**82.93 ± 2.36^{c}**	**70.76 ± 3.00^{d}**	**65.41± 1.88^{de}**	**62.82 ± 0.84^{e}**
Daidzein	ND	3.13 ± 0.21^{a}	4.92± 0.35^{b}	6.44 ± 0.56^{bc}	7.80 ± 0.59^{c}	10.54 ± 0.65^{d}
Glycitein	ND	0.86 ± 0.12^{a}	1.02± 0.13^{a}	1.63 ± 0.21^{b}	2.86 ± 0.17^{c}	2.72 ± 0.32^{c}
Genistein	4.50 ± 0.32^{a}	21.12 ± 1.35^{b}	27.89± 1.65^{c}	30.92 ± 2.31^{c}	32.48 ± 2.34^{c}	32.37 ± 2.14^{c}
Biochanin A	ND	ND	ND	ND	ND	ND
Formononetin	ND	ND	ND	ND	ND	ND
Total of aglycones	**4.50 ± 0.32^{a}**	**25.11± 1.44^{b}**	**33.83 ± 1.87^{c}**	**38.99 ± 1.96^{cd}**	**43.13 ± 3.10^{de}**	**45.63 ± 1.18^{e}**

Results expressed as mean ± standard error (n=3). Statistical analysis by means of one-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different (P>0.05) ND: Not detected (the isoflavone content which was in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method)

Table 3.4 The hydrolysis of IG in soymilk by pure β-galactosidase (2.0 U/mL)

Isoflavones (mg/100 g o freeze-dried sample)	*0 min*	*30 min*	*60 min*	*120 min*	*180 min*	*240 min*
Daidzin	14.03 ± 0.70^{a}	9.60 ± 0.95^{b}	7.98 ± 0.81^{b}	5.77 ± 0.42^{c}	5.31± 0.63^{c}	4.17± 0.58^{c}
Glycitin	6.13 ± 0.10^{a}	4.10 ± 0.56^{b}	3.04 ± 0.29^{c}	2.16 ± 0.15^{c}	0.99 ± 0.11^{d}	1.01± 0.12^{d}
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	24.49 ± 1.69^{a}	19.27 ± 1.63^{b}	17.05 ± 0.44^{bc}	14.37 ± 0.98^{cd}	12.13 ± 0.98^{d}	11.04 ± 0.78^{d}
Malonyl glycitin	3.02 ± 0.07^{a}	2.23 ± 0.16^{b}	1.10 ± 0.14^{c}	ND	ND	ND
Malonyl genistin	67.23 ± 2.02^{a}	34.86 ± 2.45^{b}	21.69 ± 1.51^{c}	18.54 ± 1.22^{cd}	13.66 ± 0.57^{de}	11.78 ± 0.92^{e}
Acetyl daidzin	6.41 ± 0.19^{a}	5.96 ± 0.87^{a}	6.05 ± 0.45^{a}	4.99 ± 0.56^{ab}	4.35 ± 0.35^{bc}	3.07 ± 0.21^{c}
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	27.50 ± 1.63^{a}	16.85 ± 0.56^{b}	16.53 ± 1.36^{b}	15.54 ± 0.81^{b}	15.51 ± 0.81^{b}	14.36 ± 0.25^{b}
Total of IG	**148.81 ± 2.88^{a}**	**92.87 ± 2.02^{b}**	**73.44 ± 1.62^{c}**	**61.37 ± 4.14^{d}**	**51.95 ± 1.61^{e}**	**45.43 ± 0.68^{e}**
Daidzein	ND	5.96 ± 0.71^{a}	7.57 ± 0.86^{a}	11.41 ± 0.74^{b}	13.10 ± 0.12^{bc}	15.28 ± 1.21^{c}
Glycitein	ND	2.72 ± 0.19^{a}	2.76 ± 0.47^{a}	3.32 ± 0.26^{a}	3.65 ± 0.31^{a}	3.67 ± 0.41^{a}
Genistein	4.50 ± 0.32^{a}	26.56 ± 1.83^{b}	29.05 ± 1.37^{bc}	34.97 ± 2.11^{cd}	37.14 ± 2.69^{d}	38.56 ± 2.95^{d}
Biochanin A	ND	ND	ND	ND	ND	ND
Formononetin	ND	ND	ND	ND	ND	ND
Total of aglycones	**4.50 ± 0.32^{a}**	**35.24 ± 1.31^{b}**	**39.38 ± 2.70^{b}**	**49.70 ± 1.63^{c}**	**53.89 ± 2.26^{cd}**	**57.51 ± 1.33^{d}**

Results expressed as mean ± standard error (n=3). Statistical analysis by means of one-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different (P>0.05) ND: Not detected (the isoflavone content which was in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method)

Table 3.5 The hydrolysis of IG in soymilk by pure β-galactosidase (4.0 U/mL)

Isoflavones (mg/100 g of freeze-dried sample)	*0 min*	*30 min*	*60 min*	*120 min*	*180 min*	*240 min*
Daidzin	14.03 ± 0.70^{a}	8.64 ± 0.95^{b}	6.48 ± 0.51^{c}	2.98 ± 0.14^{d}	2.68 ± 0.33^{d}	2.50 ± 0.18^{d}
Glycitin	6.13 ± 0.10^{a}	1.21 ± 0.23^{b}	0.81 ± 0.11^{b}	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	24.49 ± 1.69^{a}	18.91 ± 1.21^{b}	16.36 ± 0.96^{b}	7.49 ± 0.32^{c}	3.34 ± 0.15^{d}	1.23 ± 0.11^{d}
Malonyl glycitin	3.02 ± 0.07^{a}	2.00 ± 0.15^{b}	ND	ND	ND	ND
Malonyl genistin	67.23 ± 2.02^{a}	34.13 ± 1.11^{b}	22.06 ± 1.22^{c}	13.95 ± 0.25^{d}	13.40 ± 0.67^{d}	13.10 ± 0.56^{d}
Acetyl daidzin	6.41 ± 0.19^{a}	5.00 ± 0.36^{bc}	5.02 ± 0.51^{bc}	5.09 ± 0.75^{ab}	4.68 ± 0.32^{bc}	3.68 ± 0.15^{c}
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	27.50 ± 1.63^{a}	16.45 ± 1.53^{b}	15.80 ± 0.91^{b}	14.82 ± 0.98^{b}	13.94 ± 0.82^{b}	13.50 ± 0.45^{b}
Total of IG	**148.81 ± 2.88^{a}**	**86.34 ± 3.18^{b}**	**66.53 ± 4.22^{c}**	**44.33 ± 2.44^{d}**	**38.04 ± 2.29^{de}**	**34.01± 1.45^{e}**
Daidzein	ND	7.73 ± 0.76^{a}	8.90 ± 0.63^{a}	15.26 ± 0.89^{b}	17.46 ± 0.11^{bc}	18.75 ± 0.68^{c}
Glycitein	ND	2.89 ± 0.23^{a}	3.53 ± 0.19^{ab}	3.83 ± 0.23^{b}	3.86 ± 0.21^{b}	3.95 ± 0.22^{b}
Genistein	4.50 ± 0.32^{a}	27.37 ± 1.96^{b}	32.88 ± 2.11^{c}	40.02 ± 2.98^{d}	40.08 ± 2.50^{d}	40.52 ± 1.52^{d}
Biochanin A	ND	ND	ND	ND	ND	ND
Formononetin	ND	ND	ND	ND	ND	ND
Total of aglycones	**4.50 ± 0.32^{a}**	**37.99 ± 2.56^{b}**	**45.31± 1.67^{c}**	**59.11 ± 2.32^{d}**	**61.40 ± 2.18^{d}**	**63.22 ± 2.42^{d}**

Results expressed as mean ± standard error (n=3). Statistical analysis by means of one-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different (P>0.05) ND: Not detected (the isoflavone content which was in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method)

Table 3.6 The hydrolysis of IG in soymilk by pure β-glucosidase (0.5 U/mL)

Isoflavones (mg/100 g of freeze- dried sample)	*0 min*	*30 min*	*60 min*	*120 min*	*180 min*	*240 min*
Daidzin	14.03 ± 0.70	ND	ND	ND	ND	ND
Glycitin	6.13 ± 0.10	ND	ND	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	24.49 ± 1.69^{a}	4.47 ± 0.52^{b}	2.53 ± 1.08^{bc}	1.68 ± 0.15^{bc}	0.41 ± 0.05^{c}	ND
Malonyl glycitin	3.02 ± 0.07^{a}	1.82 ± 0.12^{b}	1.60 ± 0.85^{bc}	1.60 ± 0.14^{bc}	0.52 ± 0.03^{c}	ND
Malonyl genistin	67.23 ± 2.02^{a}	13.36 ± 1.02^{b}	13.23 ± 1.03^{b}	12.14 ± 0.96^{b}	12.32 ± 1.18^{b}	11.51± 1.09^{b}
Acetyl daidzin	6.41 ± 0.19	ND	ND	ND	ND	ND
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	27.50 ± 1.63^{a}	17.41 ± 1.07^{b}	17.01 ± 1.29^{b}	16.64 ± 1.12^{b}	10.23 ± 0.95^{c}	8.33 ± 0.56^{c}
Total of IG	**148.81 ± 2.88^{a}**	**37.06 ± 1.45^{b}**	**34.37 ± 2.09^{b}**	**32.06 ± 0.13^{b}**	**23.49 ± 2.15^{c}**	**19.84 ± 1.65^{c}**
Daidzein	ND	19.60 ± 1.08^{a}	20.28 ± 1.63^{a}	21.21 ± 1.32^{a}	20.83 ± 1.17^{a}	22.12 ± 1.54^{a}
Glycitein	ND	2.93 ± 1.11^{a}	3.23 ± 0.23^{a}	3.41 ± 0.31^{a}	3.71 ± 0.25^{a}	3.65 ± 0.35^{a}
Genistein	4.50 ± 0.32^{a}	40.28 ± 2.15^{b}	40.62 ± 2.56^{b}	42.49 ± 2.25^{bc}	45.02 ± 2.28^{bc}	47.93 ± 2.29^{c}
Biochanin A	ND	ND	ND	ND	ND	ND
Formononetin	ND	ND	ND	ND	ND	ND
Total of aglycones	**4.50 ± 0.32^{a}**	**62.81 ± 4.34^{b}**	**64.13 ± 3.96^{b}**	**67.11 ± 3.26^{b}**	**69.56 ± 3.20^{b}**	**73.70 ± 4.18^{b}**

Results expressed as mean ± standard error (n=3). Statistical analysis by means of one-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different (P>0.05) ND: Not detected (the isoflavone content which was in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method)

Table 3.7 The hydrolysis of IG in soymilk by pure β-glucosidase (1.0 U/mL)

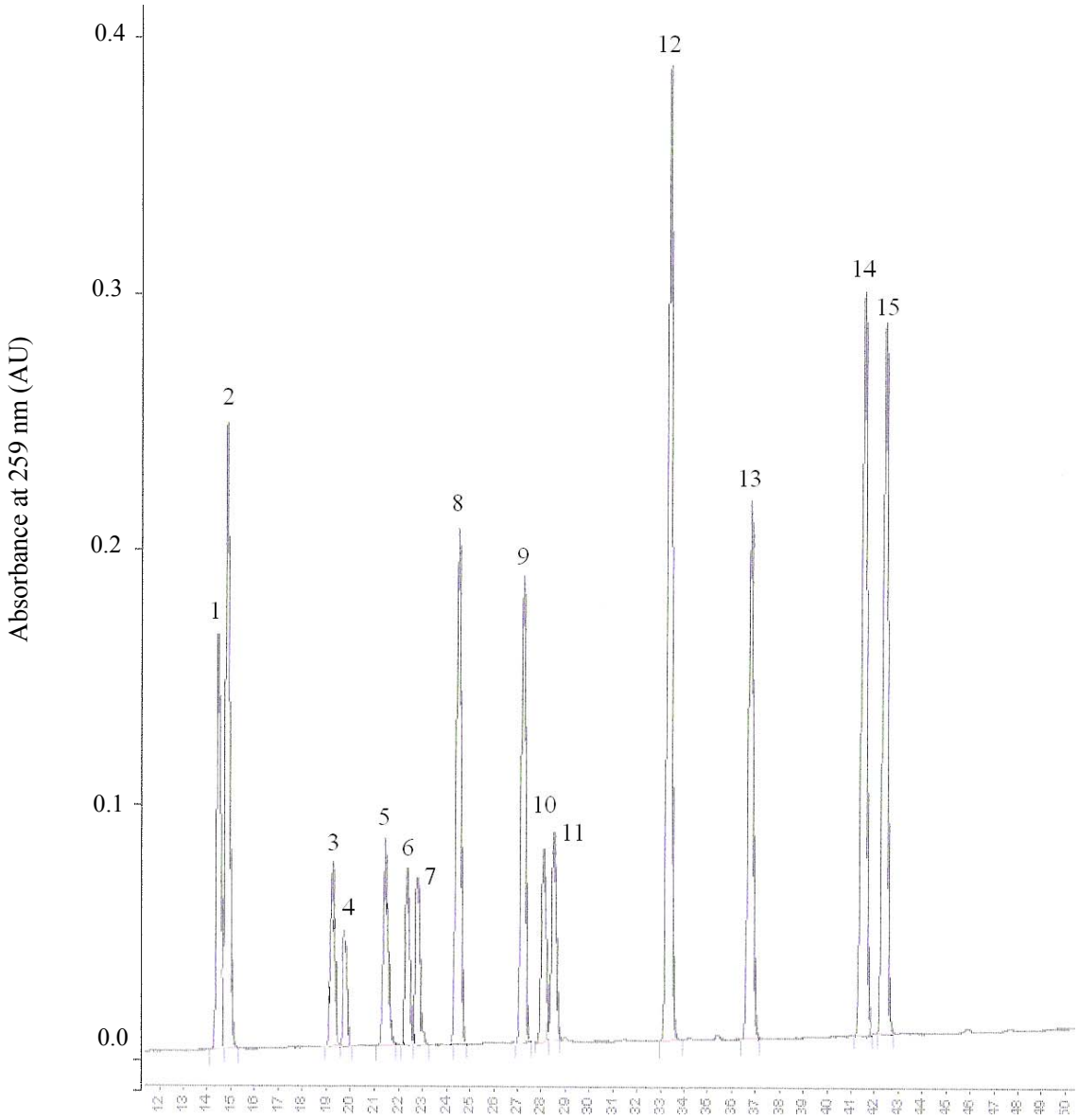
Isoflavones (mg/100 g of freeze-dried sample)	*0 min*	*30 min*	*60 min*	*120 min*	*180 min*	*240 min*
Daidzin	14.03 ± 0.70	ND	ND	ND	ND	ND
Glycitin	6.13 ± 0.10	ND	ND	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	24.49 ± 1.69^{a}	2.53 ± 0.33^{b}	2.38 ± 0.19^{b}	1.24 ± 0.15^{b}	ND	ND
Malonyl glycitin	3.02 ± 0.07^{a}	1.60 ± 0.22^{b}	1.73 ± 0.11^{b}	ND	ND	ND
Malonyl genistin	67.23 ± 2.02^{a}	12.30 ± 1.06^{b}	12.15 ± 0.97^{b}	12.66 ± 1.02^{b}	11.06 ± 0.19^{b}	10.21 ± 1.05^{b}
Acetyl daidzin	6.41 ± 0.19	ND	ND	ND	ND	ND
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	27.50 ± 1.63^{a}	14.34 ± 0.75^{b}	14.48 ± 0.85^{b}	12.32 ± 0.99^{bc}	10.54 ± 1.04^{cd}	8.45 ± 0.75^{d}
Total of IG	**148.81 ± 2.88^{a}**	**30.77 ± 2.36^{b}**	**30.74 ± 0.42^{b}**	**26.22 ± 0.18^{bc}**	**21.60 ± 1.23^{cd}**	**18.66 ± 0.30^{d}**
Daidzein	ND	20.59 ± 1.07^{a}	20.39 ± 1.23^{a}	20.30 ± 1.23^{a}	21.02 ± 1.05^{a}	22.41 ± 1.29^{a}
Glycitein	ND	3.33 ± 0.24^{a}	3.31 ± 0.24^{a}	3.31 ± 0.20^{a}	3.45 ± 0.25^{a}	3.69 ± 0.35^{a}
Genistein	4.50 ± 0.32^{a}	41.92 ± 2.85^{b}	42.20 ± 2.21^{b}	44.53 ± 2.47^{b}	45.24 ± 2.25^{b}	48.02 ± 2.29^{b}
Biochanin A	ND	ND	ND	ND	ND	ND
Formononetin	ND	ND	ND	ND	ND	ND
Total of aglycones	**4.50 ± 0.32^{a}**	**65.84 ± 4.16^{b}**	**65.90 ± 3.68^{b}**	**68.14 ± 1.44^{b}**	**69.71 ± 3.05^{b}**	**74.12 ± 0.65^{b}**

Results expressed as mean ± standard error (n=3). Statistical analysis by means of one-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different (P>0.05) ND: Not detected (the isoflavone content which was in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method)

Table 3.8 The hydrolysis of IG in soymilk by pure β-glucosidase (4.0 U/mL)

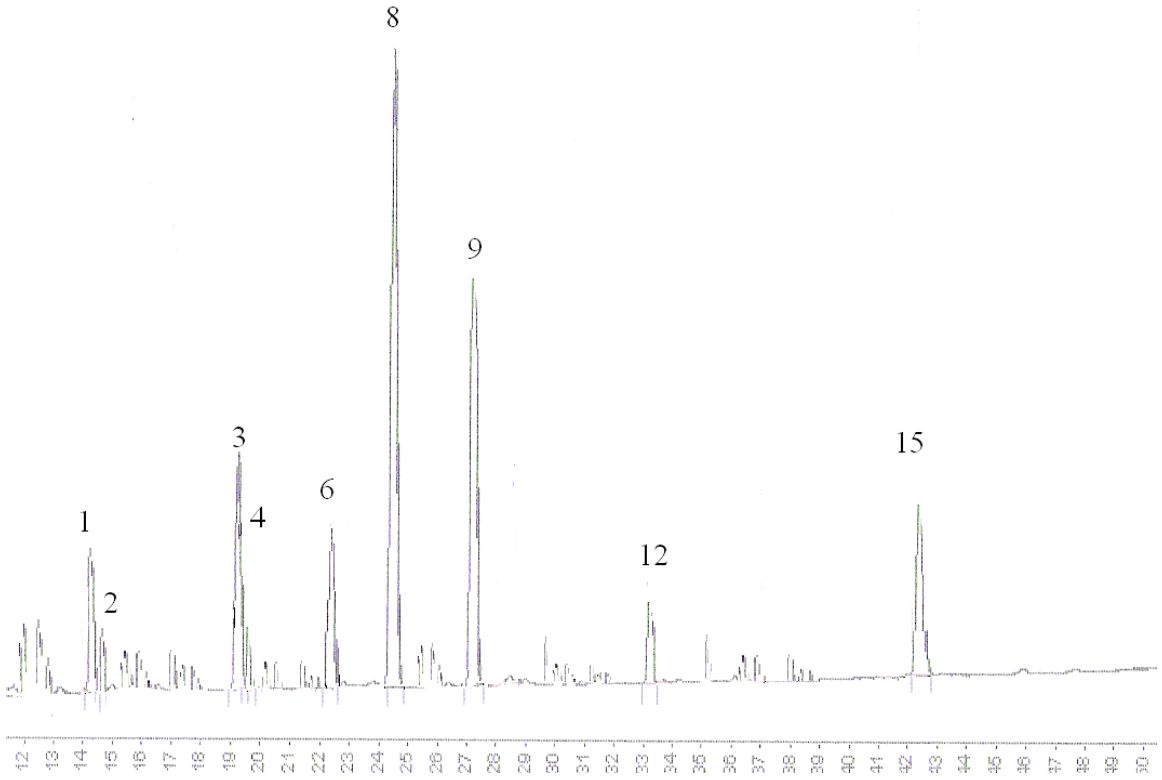
Isoflavones (mg/100 g of freeze-dried sample)	*0 min*	*30 min*	*60 min*	*120 min*	*180 min*	*240 min*
Daidzin	14.03 ± 0.70^{a}	ND	ND	ND	ND	ND
Glycitin	6.13 ± 0.10^{a}	ND	ND	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	24.49 ± 1.69^{a}	ND	ND	ND	ND	ND
Malonyl glycitin	3.02 ± 0.07^{a}	ND	ND	ND	ND	ND
Malonyl genistin	67.23 ± 2.02^{a}	11.22 ± 0.85^{b}	10.36 ± 0.81^{bc}	8.36 ± 0.54^{bc}	8.48 ± 0.36^{bc}	7.56 ± 0.69^{c}
Acetyl daidzin	6.41 ± 0.19^{a}	ND	ND	ND	ND	ND
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	27.50 ± 1.63^{a}	11.76 ± 0.98^{b}	10.77 ± 0.75^{bc}	7.77± 0.87^{cd}	5.57 ± 0.35^{de}	2.99 ± 0.42^{e}
Total of IG	**148.81 ± 2.88^{a}**	**22.98 ± 0.13^{b}**	**21.13 ± 1.56^{b}**	**16.13 ± 1.41^{c}**	**14.05 ± 0.71^{cd}**	**10.55 ± 1.11^{d}**
Daidzein	ND	20.51 ± 1.32^{a}	21.47 ± 1.14^{ab}	21.64 ± 1.20^{ab}	24.49 ± 1.24^{b}	24.20 ± 1.21^{ab}
Glycitein	ND	3.33 ± 0.23^{a}	3.63 ± 0.20^{a}	3.81± 0.15^{a}	3.75 ± 0.41^{a}	3.82 ± 0.32^{a}
Genistein	4.50 ± 0.32^{a}	45.12 ± 2.11^{b}	46.54 ± 2.63^{b}	48.02 ± 2.53^{b}	50.66 ± 3.22^{b}	51.92 ± 2.54^{b}
Biochanin A	ND	ND	ND	ND	ND	ND
Formononetin	ND	ND	ND	ND	ND	ND
Total of aglycones	**4.50 ± 0.32^{a}**	**68.96 ± 0.56^{b}**	**71.64 ± 1.29^{bc}**	**73.47 ± 1.48^{cd}**	**78.90 ± 2.39^{d}**	**79.94 ± 4.07^{d}**

Results expressed as mean ± standard error (n=3). Statistical analysis by means of one-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different (P>0.05) ND: Not detected (the isoflavone content which was in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method)



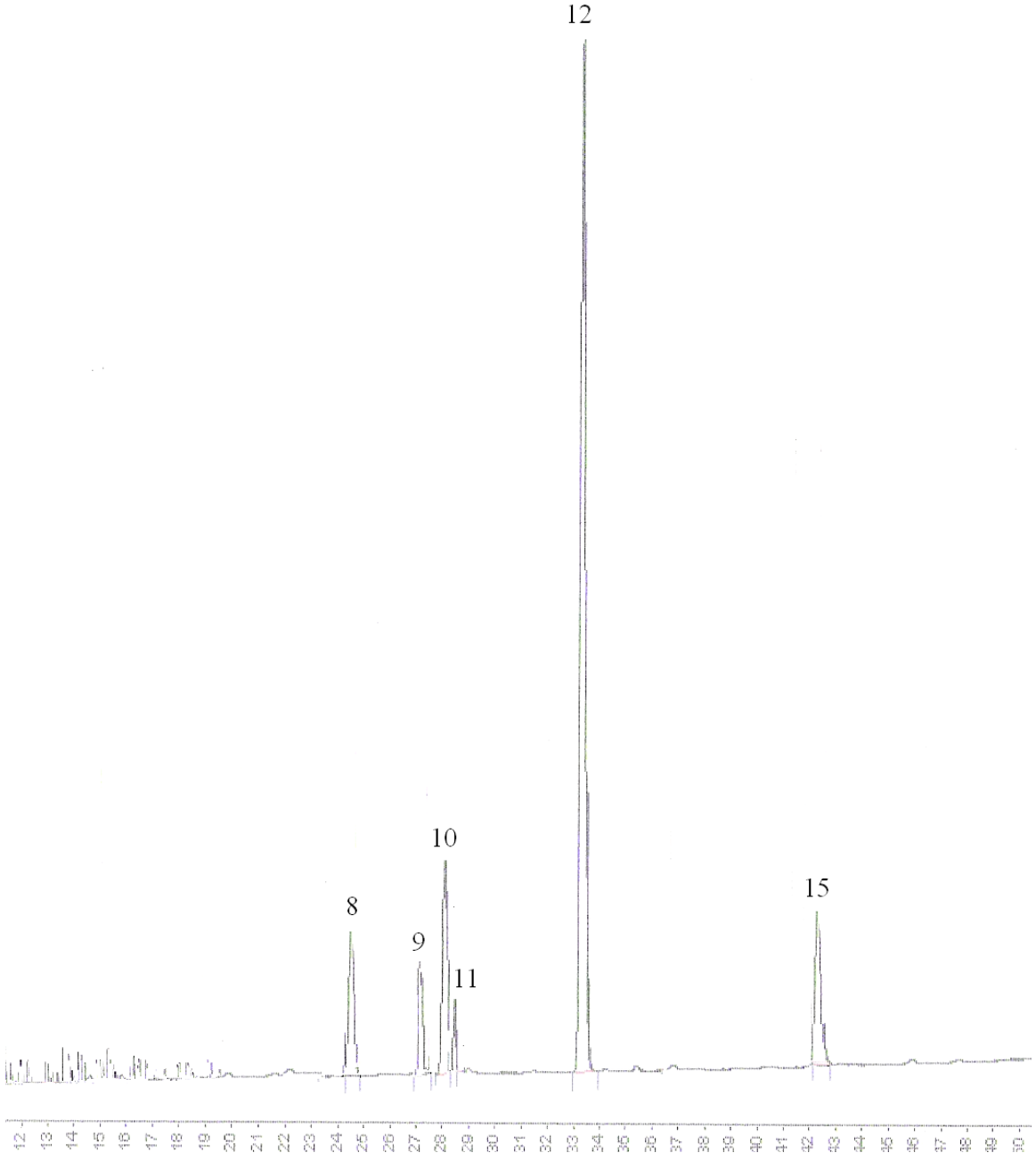
Peaks are: 1-daidzin (20 µg/mL), 2-glycitin (40 µg/mL) , 3-malonyl daidzin (8 µg/mL), 4- malonyl glycitin (4 µg/mL), 5-genistin (10 µg/mL), 6-acetyl daidzin (10 µg/mL), 7- acetyl glycitin (8 µg/mL), 8-malonyl genistin (24 µg/mL), 9- acetyl genistin (20 µg/mL), 10-daidzein (8 µg/mL), 11-glycitein (8 µg/mL), 12- genistein (32 µg/mL), 13- biochanin A (24 µg/mL), 14- formononetin (24 µg/mL), 15-flavone (20 µg/mL) (HPLC conditions: An Alltech Alltima HP C18 HL (4.6 mm i.d. x 250 mm, 5 µm particle size) column and an Alltima HP C18HL (7.5 mm x 4.6 mm internal diameter, 5 µm) guard column. Solvent A: water: phosphoric acid 1000:1 (v:v), solvent B: water: acetonitrile: phosphoric acid (200:800:1). Solvent A: 100% (0 min) → 0% (50 min) →100% (60 min). Flow rate: 0.8 mL/min).

Figure 3.1 HPLC chromatogram of 14 standard isoflavones and the internal standard



Peaks are: 1-daidzin, 2-glycitin, 3-malonyl daidzin, 4- malonyl glycitin, 6-acetyl daidzin, 8-malonyl genistin, 9- acetyl genistin, 12- genistein and 15-flavone (internal standard). (HPLC conditions as in Figure 3.1)

Figure 3.2 HPLC chromatogram of soymilk before enzymatic treatment (after autoclaving)



Peaks are: 8-malonyl genistin, 9- acetyl genistin, 10-daidzein, 11-glycitein, 12- genistein, and 15-flavone (internal standard). (HPLC conditions as in Figure 3.1)

Figure 3.3 Chromatogram of soymilk hydrolysed by β-glucosidase (4U/mL) at 240 min

Chapter 4.0

Effects of lactulose supplementation on biotransformation of isoflavone glycosides to aglycones in soymilk by probiotic organisms

This chapter has been published:

Pham, T. T., & Shah, N. P. (2008). Effect of lactulose on biotransformation of isoflavone glycosides to aglycones in soymilk by lactobacilli. **Journal of Food Science**, 73, M158-M165. (Section 4.1)

Pham, T. T., & Shah, N. P. (2008). Effect of lactulose supplementation on the growth of bifidobacteria and biotransformation of isoflavone glycosides to isoflavone aglycones in soymilk. **Journal of Agricultural and Food Chemistry**, 56, 4703-4709. (Section 4.2)

As lactobacilli and bifidobacteria are the most common genera of probiotic organisms, this chapter is divided into 2 sections. Section 4.1 and 4.2 deal with the effects of lactulose on the biotransformation of IG to IA by lactobacilli and bifidobacteria, respectively.

4.1 Effects of lactulose on biotransformation of isoflavone glycosides to aglycones in soymilk by lactobacilli

4.1.1 Introduction

Lactulose (β-D galactose 1 → 4 α-D fructose) is produced during the heat treatment of lactose as a result of an isomerisation reaction (Lobry de Bruyn-Alberda van Ekenstein rearrangement) (Chavez-Servin et al., 2006). Lactulose has been considered as a bifidogenic factor which is able to proliferate healthy intestinal microflora (Salminen & Salminen, 1997; Gonzales, Naranjo, Malec & Vigo, 2003). Lactulose was also reported to enhance the β-glucosidase and β-galactosidase activities of intestinal microflora including lactobacilli and bifidobacteria (Juskiewicz & Zdunczyk, 2002). In chapter 3.0, it was demonstrated that both of these enzymes were able to hydrolyse inactive isoflavone glycosides (IG) to isoflavone aglycones (IA), which are biologically active forms. The IA group includes daidzein, glycitein, genistein, biochanin A and formononetin (Hughes et al., 2003). As the chemical structure of IA is similar to that of estrogen, they are also classified as phytoestrogens as they are able to bind to estrogen receptor sites and therefore mimic the function of estradiol and relieve menopausal symptoms (Setchell & Cassidy, 1999). However, in nature as well as in non-fermented soy products, IA comprise a minor fraction (1.6% - 16.1%) of total isoflavone compounds ranging from 0.5 to 1.7 mg/g (King & Bignell, 2000). To achieve health benefits, the amount of IA required is 30 - 40 mg/day (Malnig & Brown, 2007). Although IG are hydrolysed to IA in the gastro-intestinal tract by gut microflora, the rate of hydrolysis varies with an individual and remained unclear (Sugano, 2005; Hughes et al., 2003). The natural sources of isoflavones are soybean, lentils, chickpeas

and red clover. Therefore, it is important to provide food with a considerable amount of IA.

To transform IG to IA, the β-glucosidic linkage between a β-glycoside and an isoflavone aglycone in an isoflavone glycoside molecule must be cleaved. Several groups of probiotic organisms have been used to convert IG to IA in soymilk (Chien et al., 2006; Otieno et al., 2006a; Tsangalis et al., 2002; Wei et al., 2007). However, the biotransformation rate of IG to IA by probiotic bacteria in general was considerably low in fermented soymilk and β-glucosidase was claimed to be the only enzyme responsible for the biotransformation (Chien et al., 2006; Tsangalis et al., 2002). Only 6.4% of the total IG in soymilk was fermented by *B. longum* after 32 h of fermentation at 37 ^{o}C (Chien et al., 2006). However, it is now realized that β-galactosidase is also responsible for the biotransformation of IG to IA (Pham & Shah, 2009a). Thus, in order to enhance the biotransformation level, soymilk (SM) could be supplemented with lactulose, which is reported to enhance β-glucosidase and β-galactosidase activities (Juskiewicz & Zdunczyk, 2002). In addition, SM is normally prepared from soy protein isolate (SPI), which is made from defatted soy flour with most of the non-protein components including fats and carbohydrates removed. Furthermore, SM prepared from SPI did not support the growth of probiotic organisms (Kamaly, 1997; Pham & Shah, 2007). As a bifidogenic factor, lactulose is expected to enhance the growth of probiotic organisms in SM supplemented with lactulose (Gonzales et al., 2003). However, there is no report about the fermentation of IG in soymilk supplemented with lactulose. Therefore, the objective of this study was to investigate the effect of lactulose on the growth of *Lactobacillus*, the predominant probiotic group, and their biotransformation ability of IG to IA in fermented soymilk.

4.1.2 Materials and Methods

4.1.2.1 Isoflavone compounds and other chemicals

Genistein, daidzein, glycitein, flavone, Carrez I, Carrez II, D-glucose and lactulose were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Daidzin, glycitin, genistin, formononetin and biochanin A were obtained from Indofine Chemical Company, Inc. (Summerville, NJ, USA). Malonyl- and acetyl- β glycosides (malonyl

daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin) were obtained from LC Labs (Woburn, MA, USA). Acetonitrile, methanol, ethanol and phosphoric acid used for HPLC were of analytical grade. Soy protein isolate SUPRO 590 was from The Solae Co. (Chatswood, NSW, Australia).

4.1.2.2 Fermentation of soymilk (SM) and soymilk supplemented with lactulose (SML) and by lactobacilli

Lactobacillus acidophilus 4461, *L. acidophilus* 4962, *L. casei* 290 and *L. casei* 2607 were obtained from the Victoria University Culture Collection (Werribee, Vic, Australia) and activated in de Mann Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK) by growing successively twice at 37 ^{o}C for 20 h. The third transfer was carried out in SM supplemented with lactulose (SML) prepared from 4% (w/v) SPI and 0.5% (w/v) lactulose or in soymilk (SM) prepared from 4% (w/v) SPI. One litre of sterile SM and SML were individually inoculated with 1% (v/v) of the active culture of *Lactobacillus* and incubated at 37 ^{o}C for 24 h. One hundred millilitres aliquots were withdrawn aseptically at 0, 6, 12, 18 and 24 h of incubation for enumeration of viable probiotic populations, determination of pH and quantification of lactulose. Fifty millilitres of the samples were freeze-dried using a Dynavac freeze-dryer (model FD 300; Rowville, Vic, Australia) for quantification of isoflavones.

4.1.2.3 Emuneration of viable of microorganisms

MRS agar was used for enumeration of probiotic organisms. Peptone water (0.15%, w/v) was used for serial dilutions. One millilitre of serially diluted samples at 0, 6, 12, 18 and 24 h was aseptically spread on to the plates and incubated at 37 ^{o}C for 3 days in an anaerobic jar (Becton Dickinson Microbiology System, Sparks, MD, USA) with a gas generating kit (Oxoid Ltd., Hampshire, UK). Colony counts between 25 - 250 were enumerated.

4.1.2.4 Determination of pH

The pH of the aliquots withdrawn at 6 h intervals during the fermentation was monitored using a microprocessor pH meter (model 8417, Hanna Instruments, Singapore) at 20 °C after calibrating with fresh pH 4.0 and 7.0 standard buffers.

4.1.2.5 Determination of lactulose concentration

Quantification of lactulose was based on Chavez-Servin, Castellote, & Lopez-Sabater (2004) with some modifications. Briefly, 10 mL of aqueous ethanol (50:50, v/v) was added to 1 mL of SM or SML and placed in a 60 ^{o}C water bath (model NB 6T-10935; Thermoline Australia, Scientific Equipments, Smithfield, NSW, Australia) until dissolved completely. To this, 250 µL of each of Carrez I and Carrez II solutions and 5 mL of acetonitrile were added and the solution was made up to 50 mL using aqueous ethanol (50:50, v/v), then filtered through Advance No. 1 filter paper, a C18 Sep-pak Plus cartridge (Waters, Milford, MA, USA) and a 0.45 µm nylon filter (Phenomenex, Lane Cove, NSW, Australia) and then injected into the HPLC system. Instrument and HPLC conditions included an Alltech Alltima (Deerfield, IL, USA) Prevail-Carbohydrate ES (4.6 x 250) mm, a 5 µm particle size column and a Hewlett Packard 1100 series HPLC (Agilent Technologies, Forest Hill, Vic, Australia) with an auto sampler, a quaternary pump, an Alltech light-scattering detector Varex MK III ELSD, a vacuum degasser and a thermostatically controlled column compartment. The injection volume was 20 µL. The mobile phase for isocratic HPLC was acetonitrile: water (70:30, v/v). The flow rate was 0.8 mL/min. Standard solutions for calibration curve were based on five lactulose working solutions prepared by diluting pure lactulose with methanol (50%, v/v) at various concentrations between 50 µg/mL to 500 µg/mL .

4.1.2.6 Determination of isoflavone contents

The method of determination of isoflavone contents is descried in Chapter 3.0, section 3.2.5 and 3.2.6. The biotransformation of IG to IA was defined as percentage of IG hydrolysed and was calculated as follows:

$$\% \text{ IG hydrolysis} = \frac{\text{initial IG} - \text{residual IG}}{\text{initial IG}} \times 100$$

4.1.2.7 Statistical analysis of data

All analyses were performed in triplicate and the data were analysed using one-way analysis of variance (ANOVA) at 95% confidence intervals using Microsoft Excel Statpro as described by Allbright et al., (1999). ANOVA data with a $P < 0.05$ was classified as statistically significant.

4.1.3 Results and Discussion

4.1.3.1 Lactulose utilisation by Lactobacillus and pH changes during incubation

Table 4.1 presents the lactulose concentration in SML during incubation. The initial lactulose content in SML was 4.82 mg/mL. There was no lactulose available in SM prepared from SPI (Nutrition Data, 2007). It appeared that *L. acidophilus* 4461 utilised the highest level of lactulose during the entire incubation. At 24 h of incubation, this probiotic organism utilised 62.4% of the initial lactulose. *Lactobacillus acidophilus* 4962 and *L. casei* 290 utilised low level of lactulose and there was no significant difference ($P > 0.05$) in the amount of lactulose used by both of them during incubation. On the other hand, *L. casei* 2607 utilised an extensive amount (1.84 mg/mL) of lactulose in the last 12 h of incubation. It appears that the decrease in the pH values of SML was dependent on the amount of lactulose utilised by *Lactobacillus*. As shown in Figure 4.1, which illustrates the pH of SML and SM fermented by the probiotic organisms, all the four *Lactobacillus* strains decreased pH in SML steadily during the fermentation. The pH decreased by *L. acidophilus* 4461 in SML was the lowest (4.00). *Lactobacillus acidophilus* 4962 utilised the least amount of lactulose, as a result the pH remained the highest (5.00) after 24 h of incubation (Figure 4.1). On the contrary, the pH of SM was only slightly reduced by all the four probiotic organisms during the fermentation. After 24 h of incubation, the pH remained at 6.15, 6.29, 6.36 and 6.31 in SM fermented by *L. acidophilus* 4461, *L. acidophilus* 4962, *L. casei* 290 and *L. casei*

2607, respectively. The pH values in SM stabilized after 12 h of incubation. Tsangalis & Shah (2004) also reported the pH of SM prepared from SPI to be high at 5.99 after 24 h of incubation by *B. animalis* Bb12. High pH in fermented products is undesirable as microbial spoilage may easily occur. However, the pH dropped to a favourable range between 4.00 and 5.00 in SML after 24 h of incubation. Hence, lactulose appeared to play a key role in decreasing pH. Lactulose is also reported to decrease the pH of infant formula based on soy fermented by probiotic organisms (Dubey & Mistry, 1996).

4.1.3.2 Viable counts of Lactobacillus during incubation

Table 4.2 shows the viable counts of lactobacilli in SML and SM. The viable counts of all the four lactobacilli were significantly higher ($P < 0.05$) in SML than those in SM during the entire incubation. The inoculated cells (log CFU/mL) of each individual *Lactobacillus* strain for both SML and SM were not significantly different ($P > 0.05$) at 0 h (Table 4.2). At 24 h of incubation, the viable counts of *Lactobacillus* were in the range of 6.99 to 7.11 log CFU/mL in SM compared to 8.08 to 8.25 log CFU/mL in SML. Soymilk did not appear to support the growth of *Lactobacillus* possibly due to low amount (less than 1%) of simple carbon compounds in SPI including sucrose, raffinose and stachyose, which have been removed during processing (Nutrition Data, 2007). However, the viable counts of *Lactobacillus* remained insignificant difference ($P > 0.05$) in the last 12 h of incubation in SM. The mild acidic condition of SM during the fermentation (pH 6.15 – 6.80) that was still in a favourable range for the growth of *Lactobacillus* could be responsible for maintaining the viability of the probiotic organisms (Shah, 2006). On contrary, the viable counts of all the four *Lactobacillus* strains decreased significantly ($P < 0.05$) at the end of incubation (24 h) in SML since the pH of the medium was relatively low and close to the tolerable limit for most lactobacilli (Shah, 2006). Salminen and Salminen (1997) also reported that lactulose promoted the growth of *L. acidophilus* in colon. Similarly, lactulose stimulated the growth of all 26 probiotic organisms in MRS broth including both *L. acidophilus* and *L. casei* in the study of Kneifel et al. (2000).

4.1.3.3 Biotransformation of IG to IA by Lactobacillus in SML and SM

Tables 4.3 to 4.6 show the biotransformation of IG to IA in SML and SM by the four *Lactobacillus* strains. The moisture content of freeze-dried samples ranged from 1.9 - 2.0%. The isoflavone concentrations were calculated back to dry basis (mg/100 g of freeze-dried sample). There were no significant differences ($P > 0.05$) in the moisture contents of the freeze-dried samples. Therefore, it is assumed that there was no effect of the moisture content on the quantification of isoflavone compounds. The samples (fermented soymilk (SM) and fermented soymilk supplemented with lactulose (SML) were all freeze-dried. Therefore, the amount of isoflavone compounds in 1 g of the dried matter of SML is lower than that in SM since lactulose added up the dried matter in SML. The HPLC chromatogram and the retention times of 14 standard isoflavone compounds and of flavone as the internal standard are shown in Figure 3.1. The internal standard eluted at 42 min and segregated from isoflavone compounds. The detection limit of HPLC method was approximately 10^{-8} g/mL.

In general, there were only 7 IG found in SM at 0 h. Genistein was the only IA detected in SM at 0 h at a low concentration (4.50 mg/100 g of freeze-dried SM). Biochanin A and formononetin were not detected in SM and SML during fermentation. This also suggests their glycosides forms (sissotrin and ononin, respectively) were not available in SPI. However, according to Ghosh and Fenner (1999), Ononin (biochanin A glucoside) and Sissotrin (formonenin glucoside) would be hydrolysed to biochanin A and formonenin, respectively. The initial IG in SM and SML at 0 h were 148.81 and 130.14 mg/100 g of freeze-dried samples, respectively. The lower initial level of isoflavone compounds in SML than SM was due to the addition of lactulose.

The biotransformation of IG to IA occurred at a similar level in SML and SM by *L. acidophilus* 4461 during the first 6 h of incubation (Table 4.3). However, the biotransformation level in SML was much higher than that in SM after 6 h of incubation. At the end of the incubation (24 h), 88.8% of IG in SML was transformed to IA compared to 68.2% in SM. Consequently, the amount of the bioactive forms IA in

SML increased to 65.55 mg/100 g of dried matter compared to 60.73 mg/100 g of dried matter in SM at 24 h of incubation (Table 4.3). However, lactulose appeared to have a stimulating effect on the biotransformation by *L. acidophilus* 4962 only during the last 12 h of incubation (Table 4.4). The results suggest that lactulose allowed the growth of *L. acidophilus* 4962. The biotransformation of IG to IA might be a consequence of high level of viable cells in SML as compared to SM. The hydrolysis of IG to IA in SML was enhanced by 9.6 to 15.0%. At 24 h of incubation, the IA produced in SM and SML were 54.11 and 57.65 mg/100 g of dried samples, respectively. Similarly, lactulose exhibited the stimulating effect on the biotransformation of IG to IA in SML by *L. casei* 290 and *L. casei* 2607 after 6 h of incubation (Tables 4.5 and 4.6). The biotransformation levels in SML by *L. casei* 290 and *L. casei* 2607 was higher (78.5 and 80.2%) compared to those of 67.5 and 67.3% in SM, respectively, at 24 h of incubation.

In general, the biotransformation of IG to IA occurred rapidly during the first 12 h of incubation. During the next 12 h of incubation, the biotransformation was considerably slow. However, it was not certain if β galactosidase lost its activity after 12 h of fermentation. The biotransformation of glucosides to aglycones was observed to be faster for only the first 12 hours of the incubation. After 12 hours of incubation, the acidity of the culture increased (pH drop significantly after 12h of incubation, Figure 4.1, page 69). Due to the acidic condition, which may be not favoured by the enzymes (β-galactosidase and β-glucosidase), hence the activity of them slowed down in resulting the biotransformation of IG to IA slowed as well. There was no significant difference ($P > 0.05$) between IA produced by all the four *Lactobacillus* at 12, 18 and 24 h of incubation in both SM and SML. The hydrolysis level of malonyl genistin and acetyl genistin by all the four *Lactobacillus* strains in SML was much higher than in SM as the residual β- glycosides genistein left in SM was much higher than that in SML.

Our study suggested that supplementation with lactulose enhanced the biotransformation of IG to IA extensively by all the four *Lactobacillus* as the level of the biotransformation in SML increased by 9.6 to 21.9% after 12 h of incubation (Tables 4.3 to 4.6). To utilise lactulose, *Lactobacillus* must generate β-D-galactosidase to hydrolyse the sugar molecule into two simple sugars including galactose and fructose (Moscone et al., 1999). This enzyme is able to cleave the β-glucosidic bond of IG molecule to produce IA. This is in agreement with the findings of Juskiewicz &

Zdunczyk (2002) who reported that the β-glucosidase and β-galactosidase activities of microorganisms from the gut of rats enhanced extensively when they were fed a diet rich in lactulose. Our study shows that the stimulating effect of lactulose on the biotransformation of IG to IA in SML compared to SM depended on the amount lactulose used by *Lactobacillus*. *Lactobacillus acidophilus* 4461 utilised the highest level of lactulose (3.01 mg/mL) and enhanced the biotransformation of IG to IA 20.6% at 24 h of incubation (Tables 4.1 and 4.3). Similarly, at the end of incubation, *L. casei* 2607, *L. casei* 290 and *L. acidophilus* 4962 and utilised 2.00, 0.92 and 0.86 mg/mL of lactulose and increased the biotransformation levels 12.9, 11.0 and 9.6% respectively, (Tables 4.1, 4.4, 4.5 and 4.6). However, it is still uncertain if there is a correlation between the more viability and the more conversion enzyme because the supplementation with lactulose provided the good carbohydrate source then enhanced the growth of microorganisms but the effect strongly depended on each strain. The presence of lactulose became a competitive substrate to IG (2 substrates are able to react with one enzyme). Therefore, the more lactulose was utilised, the more β-galactosidase's activity was, but it was not necessary the more biotransformation was. In addition, low pH condition in SML may have also contributed to the increase in the biotransformation level. Delmonte et al. (2006) and Mathias et al. (2006) reported that some IG was partly hydrolysed to IA in a low pH condition.

4.1.4 Conclusions

In conclusion, supplementation with lactulose supported the growth of *Lactobacillus* and hence, a higher level of biotransformation of IG to IA. Supplementation with lactulose supported the growth of *Lactobacillus* and the biotransformation of IG to IA. The viable counts of *Lactobacillus* in SML were significantly higher ($P < 0.05$) than those in SM during the entire incubation. The biotransformation of IG to IA was also significantly enhanced by 9.6 to 21.9% by all the four probiotic organisms in the presence of lactulose after 12 h of incubation. There was a good relationship between the lactulose utilisation and the pH of SML as well as the stimulating effect of the biotransformation level by all the four *Lactobacillus* strains. The fermentation of both SML and SM could be completed in 12 h, since not much biotransformation occurred

beyond this period. Since the supplementation of lactulose had the enhancing effect on the biotransformation of IG to IA by lactobacilli, it was expected the supplementation also had the positive effect by bifidobacteria. The next section will present the effects of the lactose supplementation on the biotransformation of IG to IA by bifidobacteria.

Table 4.1 Lactulose concentration (mg/mL) in SML during fermentation by *Lactobacillus* at 37 ^{o}C

Probiotic organisms	0 h	6 h	12 h	18 h	24 h
L. acidophilus 4461	4.82 ± 0.11^{Aa}	4.28 ± 0.17^{Ab}	4.14 ± 0.12^{Ab}	2.69 ± 0.14^{Ac}	1.81 ± 0.16^{Ad}
L. acidophilus 4962	4.82 ± 0.11^{Aa}	4.44 ± 0.15^{Ab}	4.20 ± 0.14^{Abc}	4.10 ± 0.09^{Bc}	3.96 ± 0.09^{Bc}
L. casei 290	4.82 ± 0.11^{Aa}	4.67 ± 0.17^{Aab}	4.28 ± 0.15^{ABb}	4.00 ± 0.12^{Bbc}	3.90 ± 0.10^{Bc}
L. casei 2607	4.82 ± 0.11^{Aa}	4.74 ± 0.10^{Ba}	4.66 ± 0.16^{Ba}	3.82 ± 0.09^{Bb}	2.82 ± 0.11^{Cc}

Results are expressed as mean ± standard error (n = 3). Data were analysed by means of one-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different (P > 0.05). Mean values in the same column with the same uppercase superscripts are not significantly different (P > 0.05). SML: Soymilk supplemented with lactulose. SM: soymilk

Table 4.2 Viable microbial counts (log CFU/mL) of *Lactobacillus* in SML and SM during 24 h fermentation at 37 ^{o}C

Fermentation time	0 h	6 h	12 h	18 h	24 h
L. acidophilus 4461					
SML	5.23 ± 0.07 ^{Aa}	6.92 ± 0.05 ^{Ab}	8.29 ± 0.02 ^{Ac}	8.35 ± 0.05 ^{Ac}	8.08 ± 0.03 ^{Ad}
SM	5.11 ± 0.12 ^{Aa}	6.56 ± 0.03 ^{Bb}	7.07 ± 0.05 ^{Bc}	7.12 ± 0.02 ^{Bc}	7.11 ± 0.03 ^{Bc}
L. acidophilus 4962					
SML	5.22 ± 0.07 ^{Aa}	7.10 ± 0.02 ^{Ab}	8.23 ± 0.02 ^{Ac}	8.32 ± 0.03 ^{Ac}	8.17 ± 0.06 ^{Ad}
SM	5.08 ± 0.05 ^{Aa}	6.90 ± 0.03 ^{Bb}	7.18 ± 0.04 ^{Bc}	7.10 ± 0.05 ^{Bc}	7.09 ± 0.04 ^{Bc}
L. casei 290					
SML	5.12 ± 0.07 ^{Aa}	7.07 ± 0.02 ^{Ab}	8.40 ± 0.02 ^{Ac}	8.45 ± 0.03 ^{Ac}	8.25 ± 0.06 ^{Ad}
SM	5.02 ± 0.09 ^{Aa}	6.28 ± 0.03 ^{Bb}	7.08 ± 0.04 ^{Bc}	7.03 ± 0.05 ^{Bc}	6.99 ± 0.07 ^{Bc}
L. casei 2607					
SML	5.18 ± 0.08 ^{Aa}	7.12 ± 0.07 ^{Ab}	8.34 ± 0.06 ^{Ac}	8.33 ± 0.05 ^{Ac}	8.18 ± 0.06 ^{Ad}
SM	5.07 ± 0.08 ^{Aa}	6.20 ± 0.03 ^{Bb}	7.22 ± 0.08 ^{Bc}	7.06 ± 0.05 ^{Bcd}	7.03 ± 0.08 ^{Bc}

Results are expressed as mean ± standard error (n = 3). Data were analysed by means of one-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different ($P > 0.05$). Mean values in the same column for a particular organism with the same uppercase letter are not significantly different ($P > 0.05$). SML: soymilk supplemented with lactulose. SM: soymilk.

Table 4.3 Biotransformation of IG to IA in SML and SM by *L. acidophilus* 4461 at 37 ^{o}C

Isoflavone (mg/100 g of freeze-dried sample)	SML					SM				
	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
Daidzin	12.52 ± 1.07^{a}	6.81± 0.75^{b}	1.68 ± 0.19^{c}	ND	ND	14.03 ± 0.70^{a}	8.23 ± 0.62^{b}	2.08 ± 0.21^{c}	ND	ND
Glycitin	5.36 ± 0.34^{a}	4.89 ± 0.36^{a}	ND	ND	ND	6.13 ± 0.10	ND	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	22.09 ± 1.58^{a}	11.16 ± 1.05^{b}	3.21± 0.29^{c}	3.26 ± 0.35^{c}	3.19 ± 0.28^{c}	24.49 ± 1.69^{a}	12.05 ± 1.04^{b}	4.23± 0.35^{c}	4.26 ± 0.31^{c}	4.19 ± 0.28^{c}
Malonyl glycitin	2.62 ± 0.25	ND	ND	ND	ND	3.02 ± 0.25^{a}	3.01 ± 0.21^{a}	ND	ND	ND
Malonyl genistin	57.83 ± 4.25^{a}	32.39 ± 3.11^{b}	7.25 ± 0.85^{c}	7.31 ± 0.54^{c}	7.08 ± 0.52^{c}	67.23 ± 2.02^{a}	28.80 ± 1.54^{b}	27.58 ± 1.65^{b}	27.71 ± 1.32^{bc}	26.02 ± 1.04^{c}
Acetyl daidzin	5.71± 0.65^{a}	5.05 ± 0.41^{a}	2.17 ± 0.19^{b}	2.08 ± 0.24^{b}	2.01± 0.27^{b}	6.41 ± 0.19^{a}	6.34 ± 0.40^{a}	3.07 ± 0.15^{b}	3.00 ± 0.24^{b}	3.01± 0.17^{b}
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acetyl genistin	24.01 ± 1.98^{a}	10.35 ± 1.00^{b}	2.39 ± 0.16^{c}	2.29 ± 0.18^{c}	2.32 ± 0.19^{c}	27.50 ± 1.63^{a}	14.70 ± 1.07^{b}	14.55 ± 1.85^{b}	14.82 ± 0.98^{b}	14.13 ± 0.88^{b}
Total IG	***130.14 ± 6.18^{a}***	***70.65 ± 1.08^{b}***	***16.70 ± 0.77^{c}***	***14.94 ± 0.23^{c}***	***14.60 ± 1.28^{c}***	***148.81 ± 2.94^{a}***	***73.13 ± 0.70^{b}***	***51.51 ± 4.21^{c}***	***49.79 ± 2.23^{c}***	***47.35 ± 1.81^{c}***
Daidzein	ND	8.42 ± 0.65^{a}	17.78 ± 1.32^{b}	18.65 ± 1.03^{b}	18.75 ± 0.98^{b}	ND	8.42 ± 0.71^{a}	17.89 ± 1.72^{b}	18.70 ± 1.24^{b}	19.03 ± 1.55^{b}
Glycitein	ND	0.70 ± 0.10^{a}	4.36 ± 0.29^{b}	4.35 ± 0.25^{b}	4.35 ± 0.35^{b}	ND	2.75 ± 0.15^{a}	3.71 ± 1.54^{a}	3.87 ± 0.25^{a}	3.79 ± 0.21^{a}
Genistein	3.95 ± 0.45^{a}	23.45 ± 1.57^{b}	42.31 ± 2.47^{c}	42.42 ± 2.65^{c}	42.45 ± 3.11^{c}	4.50 ± 0.32^{a}	30.86 ± 2.10^{b}	33.09 ± 1.85^{b}	33.28 ± 2.14^{b}	37.91± 2.17^{c}
Total aglycones	***3.95 ± 0.45^{a}***	***32.57 ± 2.12^{b}***	***64.45 ± 1.44^{c}***	***65.42 ± 1.87^{c}***	***65.55 ± 3.74^{c}***	***4.50 ± 0.32^{a}***	***42.03 ± 2.66^{b}***	***54.69 ± 2.03^{c}***	***55.85 ± 3.63^{c}***	***60.73 ± 3.51^{c}***
IG hydrolysed (%)	***0.0***	***45.7***	***87.2***	***88.4***	***88.8***	***0.0***	***50.9***	***65.4***	***66.5***	***68.2***

Results are expressed as mean ± standard error (n = 3). Data were analysed by means of one-way ANOVA. Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. ND: Not detected (the isoflavone content which was in 1 g freeze- dried sample used to extract isoflavones with an injection volume of 20 µL was lower than the detection limit of the method). SML: soymilk supplemented with lactulose. SM: soymilk. IG: isoflavone glycosides

Table 4.4 Biotransformation of IG to IA in SML and SM by *L. acidophilus* 4962 at 37 ^{o}C

Isoflavone (mg/100 g of freeze-dried sample)	SML					SM				
	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
Daidzin	12.52 ± 1.07^{a}	11.65 ± 0.86^{a}	3.71 ± 0.42^{b}	3.51 ± 0.29^{b}	3.44 ± 0.45^{b}	14.03 ± 0.70^{a}	13.95 ± 1.03^{a}	4.01 ± 0.48^{b}	3.72 ± 0.30^{b}	3.64 ± 0.32^{b}
Glycitin	5.36 ± 0.34^{a}	5.09 ± 0.48^{a}	2.33 ± 0.19^{b}	2.09 ± 0.22^{b}	2.00 ± 0.24^{b}	6.13 ± 0.10^{a}	5.50 ± 0.45^{a}	3.02 ± 0.20^{b}	2.39 ± 0.25^{b}	2.50 ± 0.24^{b}
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	22.09 ± 1.58^{a}	16.16 ± 1.05^{b}	6.41 ± 0.54^{c}	6.45 ± 0.57^{c}	6.22 ± 0.72^{c}	24.49 ± 1.69^{a}	24.03 ± 1.52^{a}	13.72 ± 1.08^{b}	8.53 ± 0.92^{c}	7.56 ± 0.54^{c}
Malonyl glycitin	2.62 ± 0.25^{a}	2.20 ± 0.18^{a}	ND	ND	ND	3.02 ± 0.25^{a}	2.32 ± 0.20^{b}	ND	ND	ND
Malonyl genistin	57.83 ± 4.25^{a}	53.01 ± 4.25^{a}	13.33 ± 1.26^{b}	11.50 ± 0.75^{b}	10.19 ± 0.71^{b}	67.23 ± 2.02^{a}	27.12 ± 1.41^{b}	25.03 ± 1.08^{b}	25.76 ± 1.25^{b}	23.73 ± 1.87^{b}
Acetyl daidzin	5.71± 0.65^{a}	5.53 ± 0.89^{a}	3.97 ± 0.27^{ab}	3.59 ± 0.42^{b}	3.54 ± 0.25^{b}	6.41 ± 0.19^{a}	5.93 ± 0.65^{a}	4.82 ± 0.31^{ab}	3.91 ± 0.41^{b}	3.75 ± 0.25^{b}
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acetyl genistin	24.01 ± 1.98^{a}	20.59 ± 1.05^{b}	6.99 ± 0.29^{c}	6.82 ± 0.44^{c}	6.25 ± 0.31^{c}	27.50 ± 1.63^{a}	13.85 ± 0.86^{b}	13.63 ± 1.03^{b}	13.60 ± 1.07^{b}	9.27 ± 0.75^{c}
Total IG	***130.14 ± 6.18^{a}***	***114.23 ± 8.76^{b}***	***36.74 ± 2.13^{c}***	***33.96 ± 1.19^{c}***	***31.64 ± 0.68^{c}***	***148.81 ± 2.94^{a}***	***92.70 ± 4.40^{b}***	***64.23 ± 4.18^{c}***	***57.91 ± 1.76^{cd}***	***50.45 ± 1.99^{d}***
Daidzein	ND	3.21 ± 0.22^{a}	13.05 ± 1.02^{b}	13.28 ± 1.22^{b}	13.35 ± 1.64^{b}	ND	1.04 ± 0.21^{a}	13.53 ± 1.24^{b}	14.39 ± 1.08^{b}	15.21 ± 1.32^{b}
Glycitein	ND	0.39 ± 0.11^{a}	3.06 ± 0.28^{b}	3.35 ± 0.21^{b}	3.41 ± 0.33^{b}	ND	0.90 ± 0.32^{a}	3.45 ± 0.25^{b}	3.48 ± 0.25^{b}	3.53 ± 0.11^{b}
Genistein	3.95 ± 0.45^{a}	7.25 ± 0.88^{a}	37.25 ± 2.50^{b}	38.25 ± 2.56^{b}	40.89 ± 3.10^{b}	4.50 ± 0.32^{a}	32.23 ± 1.98^{b}	33.99 ± 3.04^{b}	33.50 ± 1.52^{b}	35.37 ± 2.35^{b}
Total aglycones	***3.95 ± 0.45^{a}***	***10.85 ± 0.99^{b}***	***53.36 ± 1.20^{c}***	***54.88 ± 3.57^{c}***	***57.65 ± 1.13^{c}***	***4.50 ± 0.32^{a}***	***34.17 ± 2.51^{b}***	***50.97 ± 1.55^{c}***	***51.37 ± 2.35^{c}***	***54.11 ± 1.14^{c}***
IG hydrolysed (%)	***0.0***	***12.2***	***71.8***	***73.9***	***75.7***	***0.0***	***37.7***	***56.8***	***61.1***	***66.1***

Results are expressed as mean ± standard error (n = 3). Data were analysed by means of one-way ANOVA. Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. ND: Not detected (the isoflavone content which was in 1 g freeze- dried sample used to extract isoflavones with an injection volume of 20 µL was lower than the detection limit of the method). SML: soymilk supplemented with lactulose. SM: soymilk. IG: isoflavone glycosides

Table 4.5 Biotransformation of IG to IA in SML and SM by *L. casei* 290 at 37 ^{o}C

Isoflavone (mg/100 g of freeze-dried sample)	SML					SM				
	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
Daidzin	12.52 ± 1.07^{a}	8.54 ± 0.65^{b}	1.51 ± 0.16^{c}	ND	ND	14.03 ± 0.70^{a}	12.65 ± 1.07^{a}	5.35 ± 0.53^{b}	4.32 ± 0.25^{bc}	3.15 ± 0.25^{c}
Glycitin	5.36 ± 0.34^{a}	4.61 ± 0.42^{a}	1.53 ± 0.23^{b}	1.62 ± 0.16^{b}	1.58 ± 0.25^{b}	6.13 ± 0.10^{a}	5.21± 0.32^{b}	3.82 ± 0.20^{c}	3.08 ± 0.14^{d}	2.85 ± 0.15^{d}
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	22.09 ± 1.58^{a}	15.49 ± 1.04^{b}	3.02 ± 0.21^{c}	2.99 ± 0.25^{c}	2.89 ± 0.32^{c}	24.49 ± 1.69^{a}	20.83 ± 1.54^{b}	5.04 ± 0.43^{c}	3.81 ± 0.25^{cd}	2.69 ± 0.12^{d}
Malonyl glycitin	2.62 ± 0.25^{a}	2.53 ± 0.43^{a}	ND	ND	ND	3.02 ± 0.25^{a}	2.63± 0.12^{b}	ND	ND	ND
Malonyl genistin	57.83 ± 4.25^{a}	45.04 ± 3.21^{b}	15.84 ± 0.89^{c}	15.36 ± 0.47^{c}	14.50 ± 0.54^{C}	67.23 ± 2.02^{a}	30.33 ± 2.61^{b}	27.17 ± 2.20^{b}	25.91 ± 2.01^{b}	24.74 ± 1.85^{b}
Acetyl daidzin	5.71± 0.65^{a}	5.62 ± 0.32^{a}	3.61 ± 0.31^{b}	3.01 ± 0.24^{b}	3.06 ± 0.29^{b}	6.41 ± 0.19^{a}	6.26 ± 0.52^{a}	5.84 ± 0.41^{a}	4.02 ± 0.29^{b}	3.56 ± 0.24^{b}
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acetyl genistin	24.01 ± 1.98^{a}	16.95 ± 1.26^{b}	6.26 ± 0.35^{c}	6.05 ± 0.21^{c}	6.02 ± 0.24^{c}	27.50 ± 1.63^{a}	15.83 ± 1.09^{b}	12.25 ± 1.04^{c}	12.42 ± 0.99^{bc}	11.37 ± 0.87^{c}
Total IG	***130.14 ± 6.18^{a}***	***98.78 ± 1.61^{b}***	***31.77 ± 2.15^{c}***	***29.03 ± 1.33^{c}***	***28.05 ± 1.64^{c}***	***148.81 ± 2.94^{a}***	***93.74 ± 3.81^{b}***	***59.47 ± 1.91^{c}***	***53.56 ± 1.37^{c}***	***48.36 ± 1.26^{d}***
Daidzein	ND	5.40 ± 0.48^{a}	17.20 ± 1.23^{b}	18.32 ± 1.06^{b}	18.31 ± 1.09^{b}	ND	2.48 ± 0.36^{a}	17.19 ± 1.04^{b}	18.36 ± 1.24^{b}	19.06 ± 1.24^{b}
Glycitein	ND	0.51 ± 0.24^{a}	3.20 ± 0.24^{b}	3.23 ± 0.21^{b}	3.52 ± 0.24^{b}	ND	0.62 ± 0.15^{a}	2.87 ± 0.24^{a}	2.98 ± 0.25^{a}	3.45 ± 0.21^{a}
Genistein	3.95 ± 0.45^{a}	13.99 ± 0.99^{b}	36.35 ± 2.96^{c}	37.71 ± 2.65^{c}	38.49 ± 2.65^{c}	4.5 ± 0.32^{a}	29.62 ± 1.98^{b}	33.82 ± 2.14^{b}	34.47 ± 2.41^{b}	34.95 ± 2.24^{b}
Total aglycones	***3.95 ± 0.45^{a}***	***19.90 ± 1.71^{b}***	***56.75 ± 3.95^{c}***	***59.26 ± 1.80^{c}***	***60.32 ± 1.32^{c}***	***4.5± 0.32^{a}***	***32.72 ± 2.49^{b}***	***53.88 ± 2.94^{c}***	***55.81 ± 1.42^{c}***	***57.46 ±1.21^{c}***
IG hydrolysed (%)	***0.0***	***24.1***	***75.6***	***77.7***	***78.5***	***0.0***	***37.0***	***60.0***	***64.0***	***67.5***

Results are expressed as mean ± standard error (n = 3). Data were analysed by means of one-way ANOVA. Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. ND: Not detected (the isoflavone content which was in 1 g freeze- dried sample used to extract isoflavones with an injection volume of 20 µL was lower than the detection limit of the method). SML: soymilk supplemented with lactulose. SM: soymilk. IG: isoflavone glycosides

Table 4.6 Biotransformation of IG to IA in SML and SM by *L. casei* 2607 at 37 ^{o}C

Isoflavone (mg/100 g of freeze-dried sample)	SML					SM				
	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
Daidzin	12.52 ± 1.07^{a}	10.41 ± 0.87^{b}	1.99 ± 0.15^{c}	1.21 ± 0.16^{c}	ND	14.03 ± 0.70^{a}	13.59 ± 1.09^{a}	6.60 ± 0.78^{c}	4.21± 0.25^{d}	3.15 ± 0.21^{d}
Glycitin	5.36 ± 0.34^{a}	4.91 ± 0.43^{a}	2.79 ± 0.19^{b}	2.83 ± 0.21^{b}	1.70 ± 0.15^{c}	6.13 ± 0.10^{a}	5.32 ± 0.35^{b}	4.12 ± 0.19^{c}	3.01± 0.12^{d}	2.21± 0.12^{c}
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	22.09 ± 1.58^{a}	19.95 ± 1.18^{a}	3.65 ± 0.25^{b}	3.50 ± 0.31^{b}	3.42 ± 0.29^{b}	24.49 ± 1.69^{a}	23.21 ± 1.15^{a}	5.35 ± 0.39^{b}	5.04 ± 0.25^{b}	4.78 ± 0.19^{b}
Malonyl glycitin	2.62 ± 0.25^{a}	2.33 ± 0.25^{ab}	2.01 ± 0.19^{b}	1.05 ± 0.18^{c}	ND	3.02 ± 0.25^{a}	3.00 ± 0.25^{a}	2.85 ± 0.20^{a}	2.97 ± 0.36^{a}	2.93 ± 0.30^{a}
Malonyl genistin	57.83 ± 4.25^{a}	53.21 ± 3.11^{a}	16.03 ± 1.00^{c}	13.33 ± 1.06^{c}	12.26 ± 0.98^{c}	67.23 ± 2.02^{a}	33.49 ± 2.50^{b}	28.70 ± 1.68^{bc}	23.62± 1.27^{cd}	22.15 ± 1.52^{d}
Acetyl daidzin	5.71± 0.65^{a}	4.65 ± 0.35^{ab}	3.61 ± 0.31^{b}	3.43 ± 0.56^{b}	3.39 ± 0.48^{b}	6.41 ± 0.19^{a}	6.23 ± 0.42^{a}	5.14 ± 0.41^{b}	5.00 ± 0.35^{b}	4.55 ± 0.29^{b}
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acetyl genistin	24.01 ± 1.98^{a}	22.94 ± 1.87^{a}	7.58 ± 0.29^{b}	5.11 ± 0.28^{b}	5.02 ± 0.29^{b}	27.50 ± 1.63^{a}	16.46 ± 1.19^{b}	15.57± 1.23^{b}	10.53 ± 0.98^{c}	8.83 ± 0.79^{c}
Total IG	***130.14 ± 6.18^{a}***	***118.40 ± 5.26^{b}***	***37.66 ± 1.76^{c}***	***30.46 ± 2.08^{cd}***	***25.79 ± 0.73^{d}***	***148.81 ± 2.94^{a}***	***101.30 ± 3.46^{b}***	***68.33 ± 4.69^{c}***	***54.38 ± 1.54^{d}***	***48.60 ± 0.26^{d}***
Daidzein	ND	3.06 ± 0.45^{a}	16.16 ± 1.21^{b}	17.25 ± 1.25^{b}	18.72 ± 1.61^{b}	ND	0.69 ± 0.05^{a}	15.49 ± 1.24^{b}	16.37 ± 1.28^{b}	17.59 ± 1.08^{b}
Glycitein	ND	0.45 ± 0.11^{a}	1.65 ± 0.26^{b}	2.01 ± 0.24^{b}	3.65 ± 0.32^{c}	ND	0.55 ± 0.24^{a}	1.03 ± 0.19^{a}	2.25 ± 0.19^{a}	2.62 ± 0.25^{a}
Genistein	3.95 ± 0.45^{a}	6.85 ± 0.62^{b}	35.82 ± 1.45^{c}	37.56 ± 1.61^{c}	38.39 ± 1.33^{c}	4.5 ± 0.32^{a}	27.85 ± 1.57^{b}	32.38 ± 2.12^{bc}	36.18 ± 1.95^{c}	37.16 ± 1.69^{c}
Total aglycones	***3.95 ± 0.45^{a}***	***10.36 ± 1.18^{a}***	***53.63 ± 2.92^{b}***	***56.82 ± 3.10^{b}***	***60.76 ± 3.26^{b}***	***4.5± 0.32^{a}***	***29.09 ± 1.28^{b}***	***48.90 ± 1.07^{c}***	***54.80 ± 0.86^{d}***	***57.37 ± 3.02^{d}***
IG hydrolysed (%)	***0.0***	***9.0***	***71.1***	***76.6***	***80.2***	***0.0***	***31.9***	***54.1***	***63.5***	***67.3***

Results are expressed as mean ± standard error (n = 3). Data were analysed by means of one-way ANOVA. Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. ND: Not detected (the isoflavone content which was in 1 g freeze- dried sample used to extract isoflavones with an injection volume of 20 µL was lower than the detection limit of the method). SML: soymilk supplemented with lactulose. SM: soymilk. IG: isoflavone glycosides

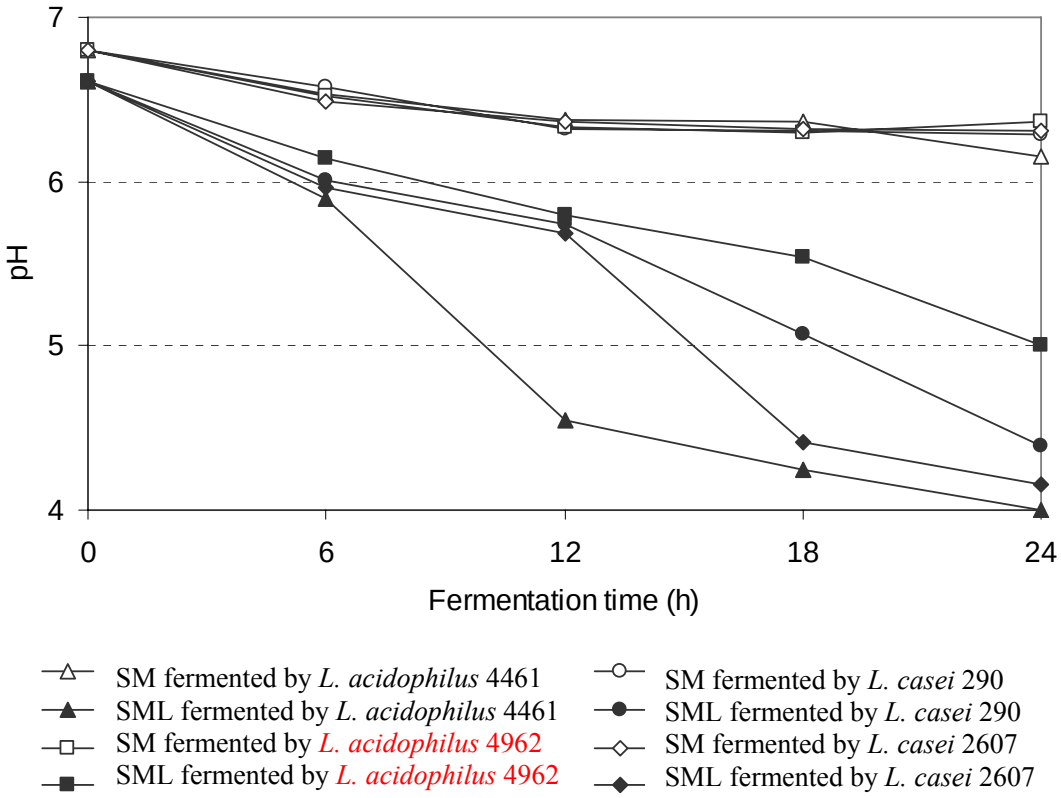


Figure 4.1 pH values of SML and SM during 24 h fermentation by *Lactobacillus* at 37 ^{o}C

Results are expressed as mean ± standard error (n = 3)

4.2 Effects of lactulose supplementation on the growth of bifidobacteria and biotransformation of isoflavone glycosides to isoflavone aglycones in soymilk

4.2.1 Introduction

Lactulose is produced during the heat treatment of lactose as a result of an isomerisation reaction (Lobry de Bruyn-Alberda van Ekenstein rearrangement) which transforms β-D-galactose 1 → 4 α-D-glucose of lactose to β-D galactose 1 → 4 α-D fructose (Chavez-Servin et al., 2006). Lactulose has been considered as a bifidogenic factor which is able to proliferate healthy intestinal microflora (Gonzales et al., 2003; Salminen & Salminen, 1997). Lactulose was also reported to enhance the β-glucosidase and β-galactosidase activities of intestinal microflora (Juskiewicz & Zdunczyk, 2002). Both of these enzymes were shown to hydrolyse isoflavone glycosides (IG), which are inactive phytochemical compounds, to isoflavone aglycones (IA), which are biologically active forms (Pham & Shah, 2009a). Although isoflavone compounds are found abundantly in soy products, soy protein isolate (SPI) is usually employed as a source of isoflavone (Soyfoods Association of North America, 2007). In addition, SPI contains approximately 85-90% protein and has a highest score of protein digestibility corrected amino acid of between 0.95 and 1.00 (Riaz, 2006). Besides, SPI can perform an excellent interaction with lipid including lipid absorption and emulsions in the food system (Riaz, 2006; Snyder & Kwon, 1987). Several methods including basic-, acidic- and enzymatic hydrolysis are reported to convert IG to IA (Delmonte et al., 2006; Mathias et al., 2006; Pham & Shah, 2009a). In the last few years, β-glucosidase producing probiotic organisms have been also used to produce IA in fermented soymilk (Chien et al., 2006; Otieno et al., 2006a; Tsangalis et al., 2002; Wei et al., 2007). These bacteria, in addition to providing this enzyme, can contribute health benefits to people consuming fermented soymilk (Shah, 2006). However, the rate of the biotransformation of IG to IA is usually low. Tsangalis et al. (2002) reported that *B. longum* transformed only 9.8% of the total isoflavone glycosides to aglycones in soymilk

after 24 h of fermentation at 37 ^{o}C. Furthermore, soymilk prepared from SPI did not support the growth of *Bifidobacterium* (Kamaly, 1997; Pham & Shah, 2007). The low level of simple carbon available in SPI (1%) may be the reason since the main carbohydrates including sucrose, raffinose and stachyose are removed during processing (Snyder & Kwon, 1987). Therefore, it is expected that the growth of probiotic organisms could be enhanced in soymilk if it is supplemented with a carbon source such as lactulose. Lactulose is also expected to stimulate the production of β-glucosidase and β-galactosidase resulting in more efficient biotransformation of IG to IA (Juskiewicz & Zdunczyk, 2002). Therefore, the objectives of this study were to investigate the influence of the supplementation with lactulose on the growth of bifidobacteria and their biotransformation ability of IG to IA in soymilk prepared from SPI.

4.2.2 Materials and Methods

4.2.2.1 Isoflavone compounds and other chemicals

Isoflavone compounds and other chemicals are described as section 4.1.2.1

4.2.2.2 Cultures and fermentation of soymilk (SM) and soymilk supplemented with lactulose (SML) by bifidobacteria

Frozen pure cultures of *Bifidobacterium animalis* subsp. *lactis* bb12 and *B. longum* 20099 were obtained from the Victoria University Culture Collection (Werribee, Vic, Australia). The two probiotic organisms were activated in De Mann Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK) (pH adjusted to 6.7 ± 0.1 using 5M NaOH) by growing successively twice at 37 ^{o}C for 20 h. The third transfer was carried out separately in SML prepared from SPI, lactulose and water (4.0, 0.5, 95.5 w/w) or in SM prepared from SPI and water (4.0, 96.0 w/w). One litre of sterile SML and SM was individually inoculated with 1% (v/v) of the active culture of probiotic organisms and anaerobically incubated at 37 ^{o}C for 24 h. One hundred milliliter aliquots were withdrawn aseptically at 0, 6, 12, 18 and 24 h of incubation for enumeration of viable probiotic populations, determination of pH and quantification of lactulose. The rest of the samples were freeze-dried using a

Dynavac freeze-dryer (model FD 300; Rowville, Vic, Australia) for quantification of isoflavones.

4.2.2.3 Determination of pH

Determination of pH is described as section 4.1.2.4

4.2.2.4 Determination of lactulose contents

Determination of lactulose is described as section 4.1.2.5

4.2.2.5 Enumeration of viable micro-organisms

Enumeration of viable is described as section 4.1.2.3

4.2.2.6 Determination of isoflavone contents

Determination of isoflavone contents is described as section 3.2.5, 3.2.6 and 4.1.2.6

4.2.2.7 Statistical analysis of data

Statistical analysis of data is described as section 4.1.2.7

4.2.3 Results and Discussion

4.2.3.1 Lactulose utilisation by bifidobacteria and the pH changes in SM and SML during incubation

Figures 4.2 and 4.3 present the lactulose utilisation by *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 in SML and the changes in pH values during the incubation, respectively. Lactulose utilisation by the two probiotic organisms increased steadily during 24 h of incubation. At the end of the incubation, *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 used 68.9 and 77.8% of the initial lactulose, respectively. The pH of SM decreased slightly from 6.80 to 6.30 by *B. animalis* subsp. *lactis* bb12 and 6.34 by *B. longum* 20099 during 24 h incubation. This result is in agreement with Tsangalis & Shah

(2004), who reported that the pH of SM prepared from SPI remained high at 5.99 after 24 h of fermentation of SM by *B. animalis*. The high pH of fermented SM may be due to lack of fermentation as a result of low levels of sugars in SPI (Nutrition Data, 2007). Garbutt (1997) indicated that sugars metabolized by fermentative organism make the medium more acidic; however, the medium remains alkaline if amino acids are used as a carbon source. High pH is undesirable for fermented product as spoilage may occur. However, it appeared that lactulose played a key role in lowering the pH of SML. The pH decreased rapidly from 6.61 to 3.90 and 4.04 in SML by *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099, respectively. Dubey & Mistry (1996) reported that the supplementation with lactulose to a soy-based formula enhanced the production of lactic and acetic acids by bifidobacteria. In our study, although *B. longum* 20099 utilised higher level of lactulose than *B. animalis* subsp. *lactis* bb12, the pH remained higher than the medium fermented by *B. animalis* subsp. *lactis* bb12. Kontula, Suihko, Wright, & Mattila-Sandholm (1999) indicated that the end products of the lactulose fermentation by lactic acid bacteria are not only organic acids but also CO_2 and ethanol, which may also affect the final pH.

4.2.3.2 Viable counts of bifidobacteria in SML and SM during incubation

Figure 4.4 shows the viable counts of the bifidobacteria in SML and SM. *Bifidobacterium animalis* subsp. *lactis* bb12 and *B. longum* 20099 showed a similar level of growth in both SM and SML during the incubation. The viable counts of both *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 in SM increased slightly from 5.70 to 7.12 and 5.90 to 6.94 log CFU/mL, respectively, after 24 h of incubation. This suggests that SM did not support their growth well, possibly due to the lack of simple sugars in SM (Riaz, 2006; Snyder & Kwon, 1987). However, the growth of the probiotic organisms significantly increased ($P < 0.05$) on supplementation with lactulose as indicated by cell counts. During 24 h of incubation, the viable counts of both *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 increased to 8.37 and 8.40 log CFU/mL, respectively. It appeared that lactulose was favoured by the probiotic organisms as they grew well in SML (Figures 4.2, 4.3 and 4.4). Lactulose also increased the viability of some *Lactobacillu*s strains including *L. casei* and *L. zeae* in the study of Desai, Powell, & Shah, (2004). Saminen & Saminen (1997) and Kontula et al. (1999) also reported that lactulose promoted the growth of *L. acidophilus*. It

has been suggested that in order to provide health benefits, the viable number of probiotic organisms must be above 10^7 cfu/g of a fermented product at the point of consumption (Ouwehan & Salminen, 1998).

4.2.3.3 Biotransformation of IG to IA in SML and SM by bifidobacteria

The moisture content of the freeze-dried samples ranged from 1.9 to 2.0%. There was no significant difference in the moisture content of the freeze-dried samples ($P > 0.05$). Therefore, it was assumed that there was no effect of the moisture content on the quantification of isoflavone compounds. The initial IG in SM and SML at 0 h were 148.81 and 130.14 mg per 100 g of freeze-dried matter, respectively. The lower initial level of the isoflavone compounds in SML was due to the supplementation with lactulose.

The biotransformation of IG to IA in SML and SM by *B. animalis* subsp. *lactis* bb12 is shown in Table 4.7. In general, the biotransformation of IG to IA occurred rapidly in the first 12 h of incubation. In the following 12 h of incubation, the level of biotransformation increased slowly. There was no significant difference ($P > 0.05$) between the IA content produced at 12, 18 and 24 h of incubation in both SM and SML by *B. animalis* subsp. *lactis* bb12. Acetyl daidzin appeared to be more stable than daidzin during the fermentation. At 18 h of incubation, daidzin was completely hydrolysed, compared to 47.5% of acetyl daidzin converting to daidzein in SML. Similarly, at 18 h of incubation in SM, 77.1% and 45.4% of daidzin and acetyl daidzin were hydrolysed, respectively. Mathias et al. (2006) reported that acetyl daidzin was fairly stable in a low pH condition. Our study showed that supplementation with lactulose extensively enhanced the biotransformation level of IG to IA during incubation. The level of biotransformation in SML ranged from 49.6% to 85.6%, which was 6.7 to 14.7% higher than that in SM. At the end of incubation, IA comprised 77.1% (63.21 mg/100 g of freeze-dried sample) compared to 58.8% (61.88 mg/100 g of freeze-dried sample) of total isoflavone compounds in SML and SM, respectively. Daidzin and acetyl daidzin were hydrolysed entirely in SML; however, they were still present in SM after 24 h of incubation.

Table 4.8 shows the biotransformation of IG to IA in SML and SM by *B. longum* 20099. Similar to *B. animalis* subsp. *lactis* bb12, the biotransformation of IG to IA occurred rapidly in the first 12 h of incubation. Although the initial level of glycitin was lower than daidzin, it was still detected at 24 h of incubation, while daidzin was completely hydrolysed in both SML and SM. This suggests that *B. longum* 20099 transformed daidzin more efficiently than glycitin. The reason might due to their chemical structures and molecular weights. Daidzin has lower molecular weight and fewer branches in its chemical structure (Figure 2.2). Due to these factors, it may enter easier to the active zone of the enzymes resulting in the faster hydrolysis. The supplementation with lactulose increased the biotransformation of IG to IA by *B. longum* 20099 from 12.8 to13.4%. However, the stimulating effect was only observed from 12 h of incubation (Table 4.8). At 6 h of incubation, IG were transformed to IA at the lower level in SML (26.3%) compared to that in SM (44.4%). At the end of incubation, IA increased from 2.9 to 69.5% of the total isoflavones in SML compared to 54.3% in SM. As regards the residual IG after 24 h of incubation, *B. animalis* subsp. *lactis* bb12 hydrolysed β-glycosides genistin better than *B. longum* 20099 did in both SM and SML, while *B. longum* 20099 hydrolysed daidzin more effectively than *B. animalis* subsp. *lactis* bb12 in SM. In general, *B. animalis* subsp. *lactis* bb12 exhibited better biotransformation of IG to IA than that of *B. longum* 20099 in both SML and SM (Tables 4.7 and 4.8). On the other hand, the lactulose utilisation did not show any relationship with the level of biotransformation of IG to IA. *Bifidobacterium animalis* subsp. *lactis* bb12 utilised lower level of lactulose than that of *B. longum* 20099, but the biotransformation level was higher during the incubation. Our data suggested that the presence of lactulose in the medium enhanced the β galactosidase activity. However, lactulose also became a competitive substrate to IG. Therefore, the more lactulose was utilised, the more β galactosidase's activity was, but it was not necessary the more biotransformation was as this strongly depended on metabolisms of each microorganism.

Tsangalis et al. (2002) reported that *Bifidobacterium pseudolongum* converted 57.8% of IG to aglycones in SM after 24 h of incubation while in the study of Chien et al. (2006), *B. longum* hydrolysed only 6.4% of IG to IA after 32 h of incubation. Therefore, the biotransformation level appeared to vary widely among probiotic organisms. Juskiewicz & Zdunczyk (2002) suggested that the β-glucosidase and β-galactosidase activities of

microorganisms from the gut of rats enhanced extensively when they were fed a diet rich in lactulose. In addition, to utilise lactulose the two strains of *Bifidobacterium* must have produced β-D-galactosidase to hydrolyse a lactulose molecule into two simple sugars including galactose and fructose. Hence, the presence of lactulose in SML may have enhanced the two enzymes produced by *Bifidobacterium*, and as the result the biotransformation of IG to IA was enhanced.

4.2.4 Conclusions

Lactulose appeared to be a favourable carbon source for both *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 as the supplementation with lactulose supported their growth. The viable counts of bifidobacteria in SML were significantly higher ($P < 0.05$) than those in SM during the entire incubation, although the presence of lactulose plays a key role in decreasing the pH values in media. The lowering of pH of SML due to supplementation with lactulose may have enhanced the biotransformation of IG to IA. The biotransformation increased up to 17.1% by the probiotic organisms in the presence of lactulose after 12 h of incubation. The fermentation of both SML and SM could be completed in 18 h, since not much biotransformation occurred beyond this period. Therefore, the lactulose supplementation had the enhancing effect on the biotransformation of IG to IA by both lactobacilli and bifidobacteria. Our results suggested that the enhancing effects by the *L. acidophilus* strains attained the highest enhancement (19.6- 20.6%), followed by the bifidobacteria (13.2 -14.6%) and the *L. casei* strains (11.0-12.9%). In addition, the lactose supplementation had the enhancing effect on each individual strain, it would have the same enhancing effect when used in combination of lactobacilli and bifidobacteria.

Table 4.7 Biotransformation of IG to IA in SML and SM by *B. animalis* subsp. *lactis* bb12 at 37 ^{o}C during 24 h incubation

Isoflavone (mg/100 g of freeze-dried sample)	SML					SM				
	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
Daidzin	12.52 ± 1.07^{a}	3.98 ± 0.32^{b}	1.89 ± 0.15^{c}	ND	ND	14.03 ± 0.70^{a}	9.34 ± 0.75^{b}	6.01 ± 0.54^{c}	3.21 ± 0.25^{d}	2.98 ± 0.21^{d}
Glycitin	5.36 ± 0.34^{a}	5.01 ± 0.54^{a}	1.88 ± 0.26^{b}	1.69 ± 0.23^{b}	ND	6.13 ± 0.10^{a}	5.50 ± 0.60^{a}	3.20 ± 0.36^{b}	1.51± 0.24^{c}	ND
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	22.09 ± 1.58^{a}	9.52 ± 0.52^{b}	7.21 ± 0.25^{bc}	5.11 ± 0.29^{cd}	4.05 ± 0.31^{d}	24.49 ± 1.69^{a}	17.04 ± 1.04^{b}	8.31 ± 0.71^{c}	6.50 ± 0.42^{cd}	5.80 ± 0.35^{d}
Malonyl glycitin	2.62 ± 0.25	ND	ND	ND	ND	3.02 ± 0.07	ND	ND	ND	ND
Malonyl genistin	57.83 ± 4.25^{a}	29.86 ± 2.12^{b}	10.21 ± 0.56^{c}	9.99 ± 0.74^{c}	9.52 ± 0.45^{c}	67.23 ± 2.02^{a}	29.35 ± 1.37^{b}	22.61 ± 1.57^{c}	20.25 ± 2.12^{c}	18.33 ± 1.04^{c}
Acetyl daidzin	5.71± 0.65^{a}	5.61 ± 0.62^{a}	3.02 ± 0.28^{b}	3.00 ± 0.32^{b}	ND	6.41 ± 0.19^{a}	6.20 ± 0.51^{a}	4.21 ± 0.36^{b}	3.50 ± 0.24^{bc}	2.98 ± 0.20^{c}
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acetyl genistin	24.01 ± 1.98^{a}	11.57 ± 0.88^{b}	5.33 ± 0.41^{c}	5.26 ± 0.39^{c}	5.21 ± 0.52^{c}	27.50 ± 1.63^{a}	17.60 ± 1.08^{b}	14.87 ± 1.21^{b}	13.85 ± 1.17^{b}	13.25 ± 1.11^{b}
Total IG	130.14 ± 6.18^{a}	65.55 ± 2.08^{b}	29.54 ± 1.09^{c}	25.05 ± 1.33^{cd}	18.78 ± 1.71^{d}	148.81 ± 2.88^{a}	85.03 ± 4.15^{b}	59.21 ± 1.61^{c}	48.82 ± 1.62^{d}	43.34 ± 2.01^{d}
Daidzein	ND	12.21 ± 1.00^{a}	17.25 ± 1.02^{b}	18.78 ± 1.32^{b}	19.45 ± 1.56^{b}	ND	6.32 ± 0.45^{a}	16.87 ± 1.25^{b}	17.75 ± 1.32^{b}	18.42 ± 1.24^{b}
Glycitein	ND	1.36 ± 0.16^{a}	3.22 ± 0.25^{b}	3.35 ± 0.32^{b}	4.01 ± 0.45^{b}	ND	2.12 ± 0.22^{a}	3.25 ± 0.25^{a}	3.95± 0.31^{a}	4.25 ± 0.54^{a}
Genistein	3.95 ± 0.45^{a}	26.75 ± 1.85^{b}	39.25 ± 2.54^{c}	39.65 ± 3.11^{c}	39.75 ± 2.96^{c}	4.50 ± 0.32^{a}	30.31± 2.46^{b}	37.97 ± 2.11^{c}	38.03 ± 2.58^{c}	39.21± 2.59^{c}
Total IA	3.95 ± 0.45^{a}	40.32 ± 3.01^{b}	59.72 ± 3.81^{c}	61.78 ± 4.11^{c}	63.21 ± 4.97^{c}	4.50 ± 0.32^{a}	38.75 ± 3.13^{b}	58.09 ± 3.61^{c}	59.73 ± 1.57^{c}	61.88 ± 3.29^{c}
IG hydrolysed (%)	0.0	49.6	77.3	80.8	85.6	0.0	42.9	60.2	67.2	70.9

Results expressed as mean ± standard error (n = 3). Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. ND: Not detected (the isoflavone content which was in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method). SML: soymilk supplemented with lactulose. SM: soymilk. IG: isoflavone glycosides. IA: Isoflavone aglycones

Table 4.8 Biotransformation of IG to IA in SML and SM by *B. longum* 20099 at 37 ^{o}C during 24 h incubation

Isoflavone (mg/100 g of freeze-dried sample)	SML					SM				
	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
Daidzin	12.52 ± 1.07^{a}	9.27 ± 1.06^{b}	4.05 ± 0.42^{c}	2.05 ± 0.42^{c}	ND	14.03 ± 0.70^{a}	6.59 ± 0.42^{b}	3.20 ± 0.24^{c}	1.50 ± 0.15^{d}	ND
Glycitin	5.36 ± 0.34^{a}	5.00 ± 0.66^{a}	2.01 ± 0.19^{b}	1.79 ± 0.21^{b}	1.59 ± 0.19^{b}	6.13 ± 0.10^{a}	5.41± 0.62^{a}	3.06 ± 0.39^{b}	2.35 ± 0.32^{bc}	1.92 ± 0.20^{c}
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	22.09 ± 1.58^{a}	16.83 ± 1.12^{b}	8.21 ± 0.75^{c}	5.11 ± 0.75^{c}	5.08 ± 0.68^{c}	24.49 ± 1.69^{a}	17.51 ± 0.37^{b}	10.65 ± 0.32^{c}	6.21± 0.70^{d}	4.61± 0.43^{d}
Malonyl glycitin	2.62 ± 0.25^{a}	1.63 ± 0.25^{b}	ND	ND	ND	3.02 ± 0.07	ND	ND	ND	ND
Malonyl genistin	57.83 ± 4.25^{a}	43.92 ± 2.54^{b}	14.29 ± 0.98^{c}	13.25 ± 0.89^{c}	11.08 ± 0.78^{c}	67.23 ± 2.02^{a}	31.49 ± 1.75^{b}	29.87 ± 1.54^{bc}	28.85 ± 1.65^{bc}	26.90 ± 1.82^{c}
Acetyl daidzin	5.71± 0.65^{a}	5.21 ± 0.54^{ab}	4.99 ± 0.51^{b}	3.20 ± 0.34^{c}	3.15 ± 0.49^{c}	6.41 ± 0.19^{a}	5.31± 0.62^{a}	5.11± 0.59^{a}	3.86 ± 0.53^{ab}	3.19 ± 0.23^{b}
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acetyl genistin	24.01 ± 1.98^{a}	14.09 ± 1.01^{b}	6.32 ± 0.35^{c}	6.21 ± 0.58^{c}	6.25 ± 0.41^{c}	27.50 ± 1.63^{a}	16.50 ± 1.06^{b}	13.45 ± 1.19^{b}	13.39 ± 1.08^{b}	13.54 ± 0.99^{b}
Total of IG	130.14 ± 6.18^{a}	95.95 ± 4.66^{b}	39.87 ± 1.66^{c}	31.61 ± 1.19^{c}	27.15 ± 1.78^{c}	148.81 ± 2.88^{a}	82.81± 2.02^{b}	65.34 ± 2.45^{c}	56.16 ± 2.27^{d}	50.16 ± 2.81^{d}
Daidzein	ND	4.68 ± 0.65^{a}	13.52 ± 1.05^{b}	17.80 ± 1.11^{c}	19.03 ± 1.02^{c}	ND	7.89 ± 1.25^{a}	14.89 ± 1.54^{a}	18.67 ± 1.29^{a}	20.40 ± 1.47^{a}
Glycitein	ND	0.69 ± 0.15^{a}	3.19 ± 0.29^{b}	3.33 ± 0.21^{b}	3.51 ± 0.29^{b}	ND	1.96 ± 0.28^{a}	3.45 ± 0.21^{a}	3.92 ± 0.27^{a}	3.98 ± 0.33^{a}
Genistein	3.95 ± 0.45^{a}	16.92 ± 1.22^{b}	37.37 ± 3.01^{c}	38.23 ± 2.65^{c}	39.41 ± 3.14^{c}	4.50 ± 0.32^{a}	29.80 ± 1.38^{b}	33.83 ± 2.14^{bc}	33.52 ± 1.78^{bc}	35.27 ± 2.50^{c}
Total of IA	3.95 ± 0.45^{a}	22.29 ± 0.42^{b}	54.08 ± 4.35^{c}	59.36 ± 3.97^{c}	61.95 ± 4.45^{c}	4.50 ± 0.32^{a}	39.65 ± 2.91^{b}	52.17 ± 0.39^{bc}	56.11 ± 2.80^{bc}	59.65 ± 3.64^{c}
IG hydrolysed (%)	0.0	26.3	69.4	75.7	79.1	0.0	44.4	56.1	62.3	66.3

Results expressed as mean ± standard error (n = 3). Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. ND: Not detected (the isoflavone content which was in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method). SML: soymilk supplemented with lactulose. SM: soymilk. IG: isoflavone glycosides. IA : Isoflavone aglycones

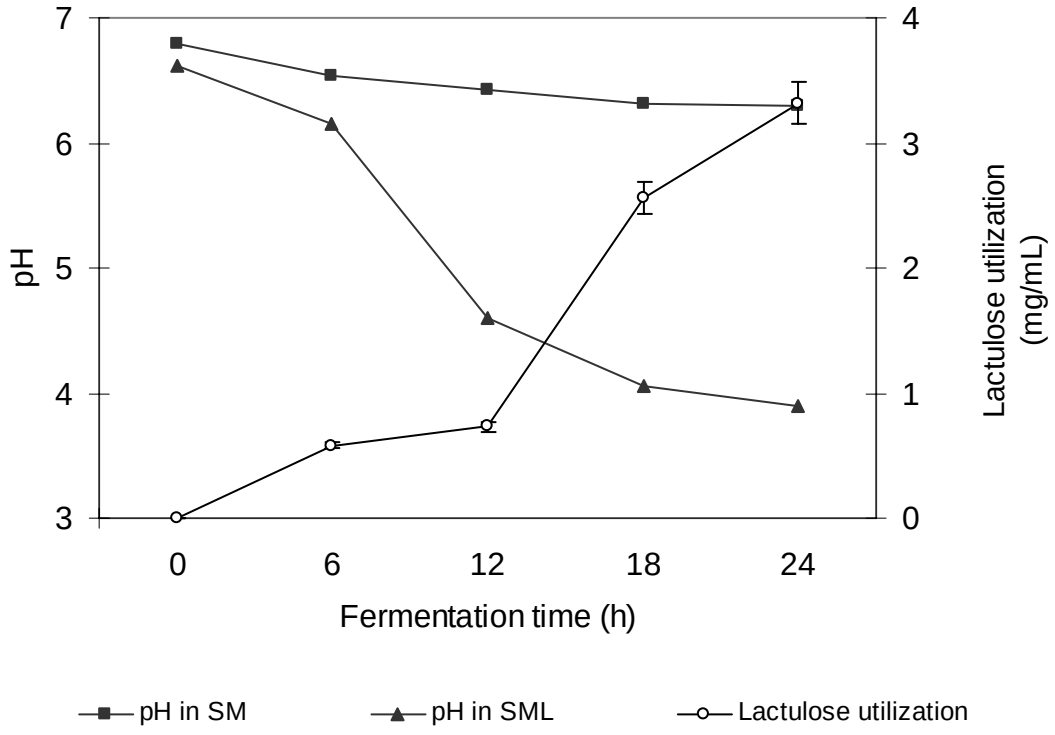


Figure 4.2 Changes in pH values in SM and SML and lactulose utilisation in SML by *B. animalis* subsp. *lactis* bb12 at 37 ^{o}C during 24 h of fermentation

Results expressed as mean ± standard error (n = 3)

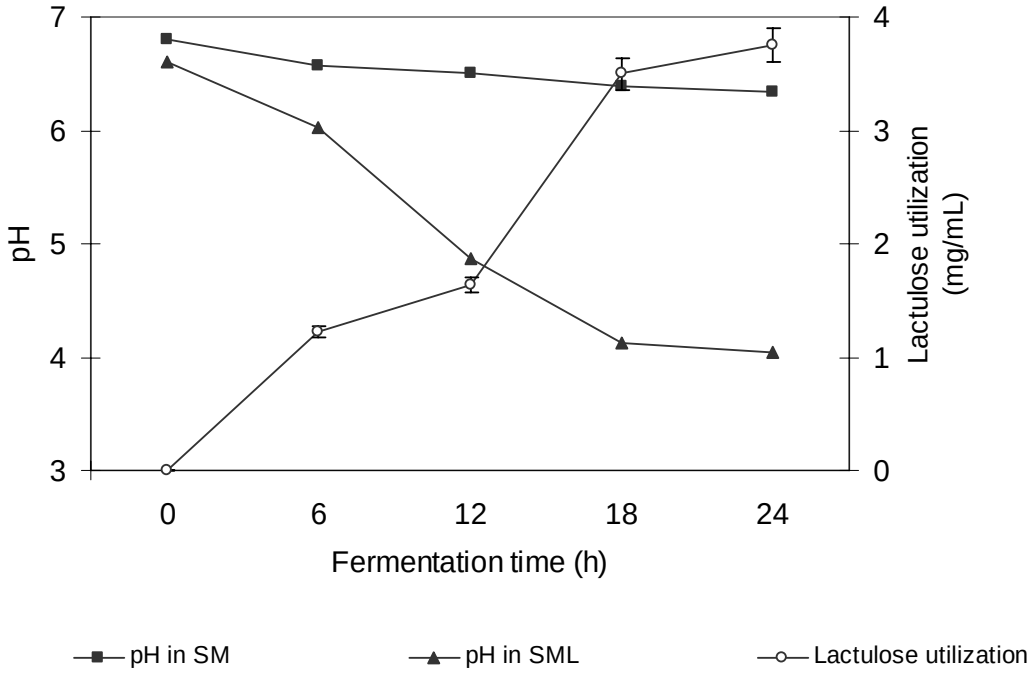


Figure 4.3 Changes in pH values in SM and SML and lactulose utilisation in SML by *B. longum* 20099 at 37 ^{o}C during 24 h of fermentation

Results expressed as mean ± standard error (n = 3)

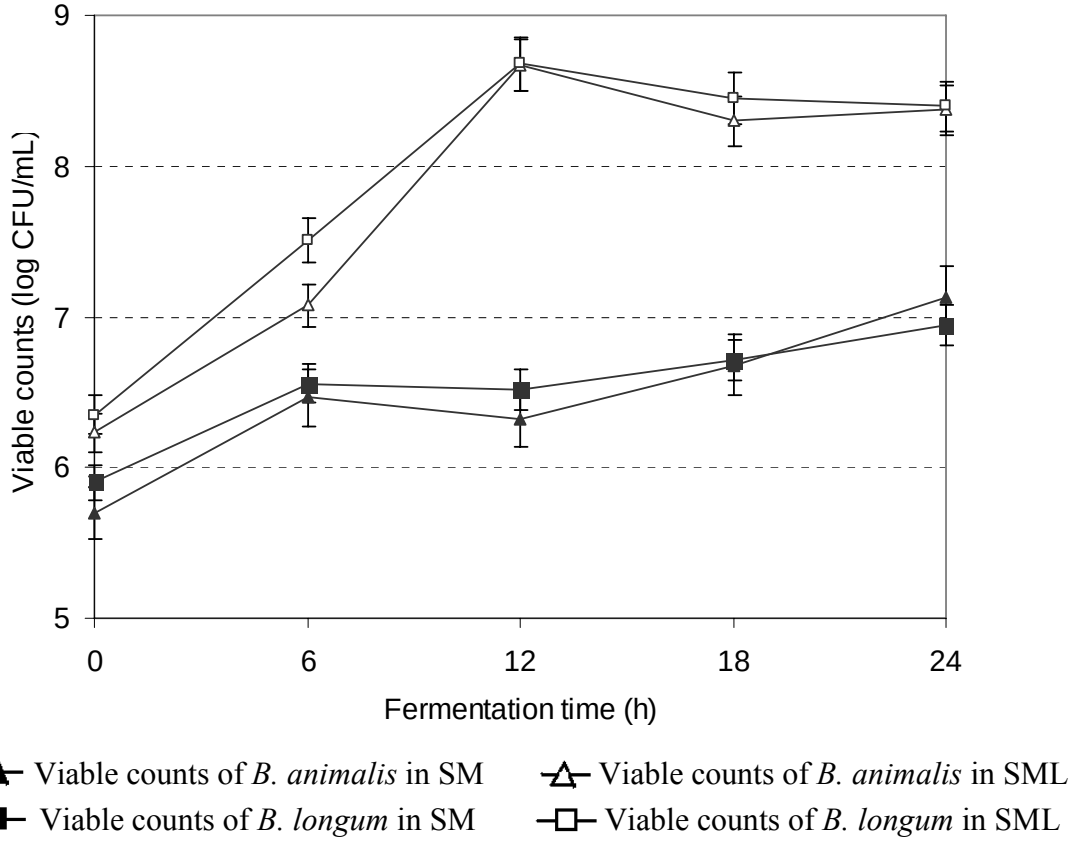
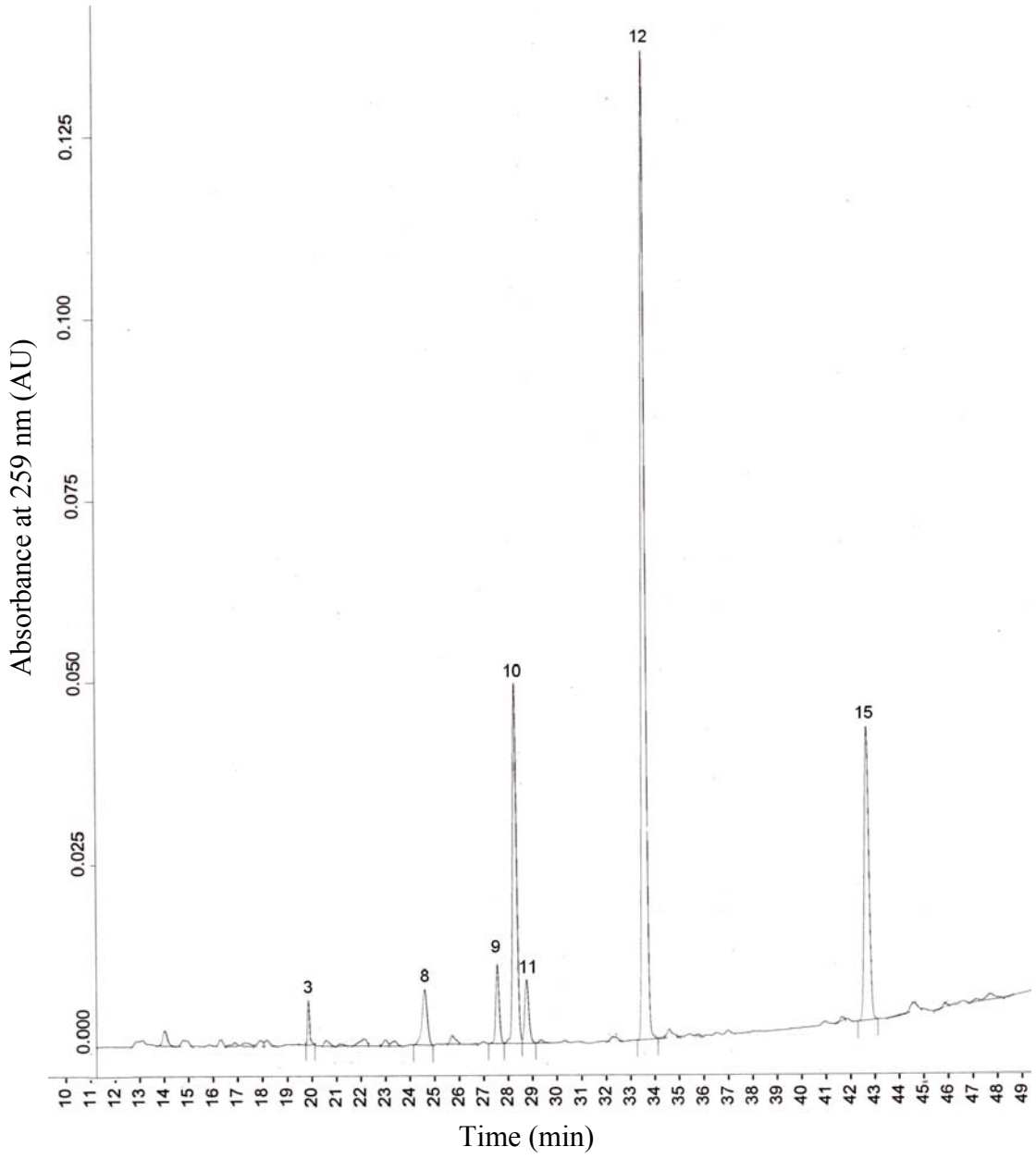


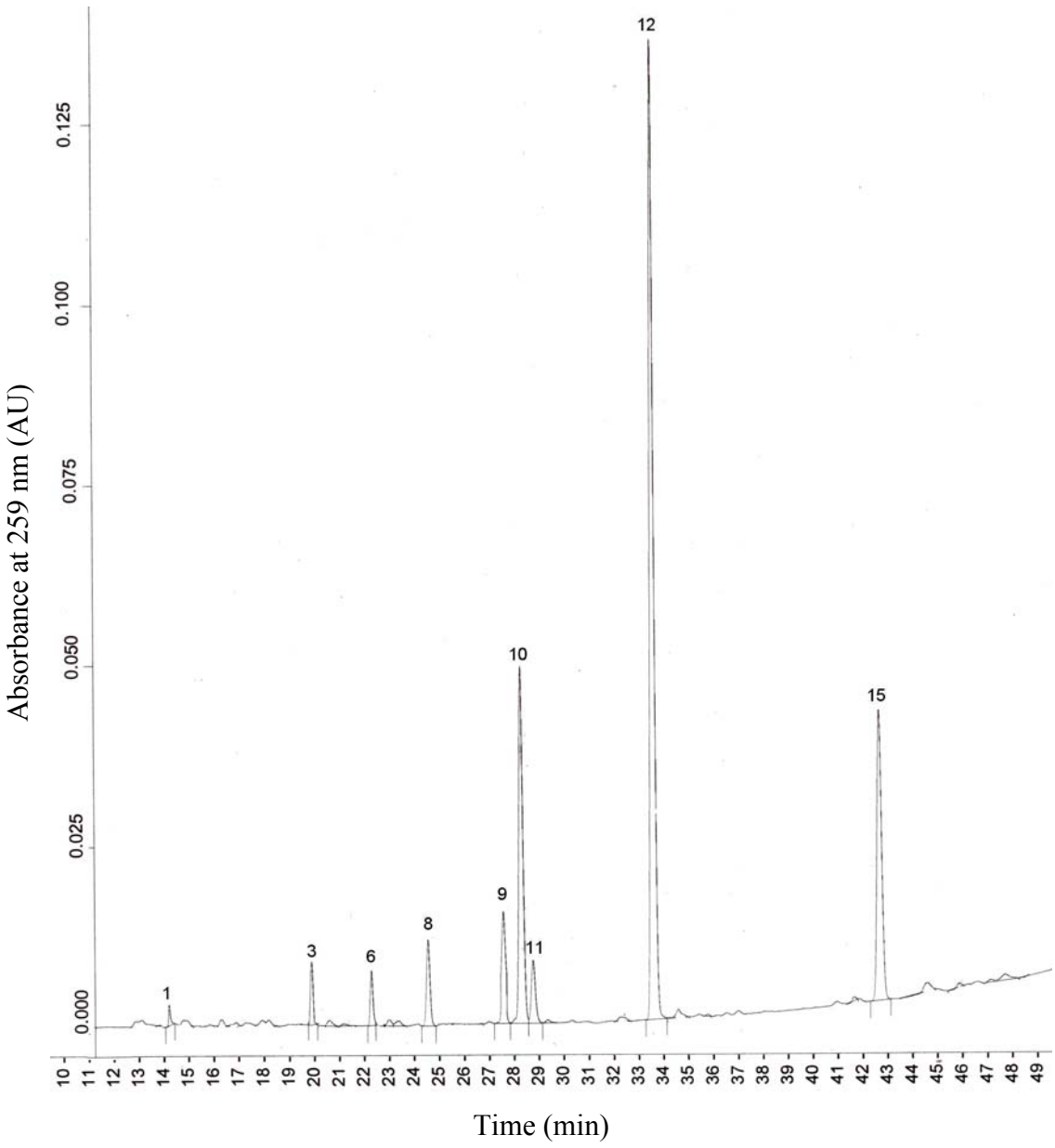
Figure 4.4 Viable counts of *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 in SM and SML during fermentation for 24 h at 37 ^{o}C

Results expressed as mean ± standard error (n = 3)



Peaks are: 3-malonyl daidzin, 8-malonyl genistin, 9- acetyl genistin, 10-daizein, 11-glycitein, 12- genistein and 15-flavone

Figure 4.5 Chromatograms of isoflavone compounds in SML at 24 h of fermentation at 37 ^{o}C by *B. animalis* subsp. *lactis* bb12



Peaks are: 1-daidzin, 3-malonyl daidzin, 6-acetyl daidzin, 8-malonyl genistin, 9- acetyl genistin, 10-daizein, 11-glycitein, 12- genistein and 15-flavone

Figure 4.6 Chromatograms of isoflavone compounds in SM at 24 h of fermentation at 37 ^{o}C by *B. animalis* subsp. *lactis* bb12

Chapter 5.0

Effects of the supplementation with skim milk powder on the biotransformation of isoflavone glycosides to aglycones in soymilk by probiotic organisms

This chapter has been published

Pham, T. T., & Shah, N. P. (2008). Skim milk powder supplementation affects lactose utilisation, microbial survival and biotransformation of isoflavone glycosides to isoflavone aglycones in soymilk by *Lactobacillus*. **Food Microbiology**, 25, 653-661. (Section 5.1)

Pham, T. T., & Shah, N. P. (2007). Biotransformation of isoflavone glycosides by *Bifidobacterium animalis* in soymilk supplemented with skim milk powder. **Journal of Food Science**, 72(8), M316 -M324. (Section 5.2)

This chapter is divided into 2 sections. Section 5.1 and 5.2 deal with the effects of skim milk powder on the biotransformation of IG to IA by the most two common of the probiotic groups including lactobacilli and bifidobacteria, respectively.

5.1 *Effects of the supplementation with skim milk powder on the biotransformation of isoflavone glycosides to aglycones in soymilk by Lactobacillus*

5.1.1 Introduction

Soy protein isolate (SPI) was isolated for the first time in 1936 by Julian, an organic chemist (Riaz, 2006). The protein digestibility corrected amino acid score for SPI is between 0.95 and 1.00, thus it is considered a complete source of protein (Riaz, 2006; Snyder & Kwon, 1987; Sugano, 2005). In addition, SPI is able to form stable emulsion and foam in fermented dairy products; hence it is used as an emulsifier (Snyder & Kwon, 1987). Due to these characteristics, SPI has been used widely in the food industry for several decades (Johnson, 1975; Riaz, 2006). Besides, SPI contains a considerable amount of isoflavones (Hughes et al., 2003). With a structural homology to human estrogens, isoflavones are considered a "natural way" to replenish the aging body's declining estrogen levels (Setchell, 1998; Setchell & Cassidy, 1999). However, the main isoflavone compounds found in SPI occur as aglycone-glycoside conjugates or isoflavone glycosides (IG) which do not possess any estrogenic activity (Hughes et al., 2003; Pham & Shah, 2007; Setchell, 1998). Five biologically active forms of isoflavone aglycones (IA), including daidzein, glycitein, genistein, biochanin A and formononetin comprise a minor fraction of isoflavone compounds in SPI (Figure 2.2) (Hughes et al., 2003). The concentration of IA is approximately 5 mg per 100 g of dried sample, which is much less than the amount required (30 - 40 mg/day) to achieve any health benefit (Malnig & Brown, 2007; Pham & Shah, 2007). Therefore, it is necessary to provide food products with a considerable amount of IA. Although IG are hydrolysed to IA in the gastro-intestinal tract, the rate of hydrolysis varies with an individual and is usually low (Sugano, 2005).

To transform IG to IA, the β-glucosidic linkage between a β-glycoside and an aglycone in IG molecule must be cleaved. Several groups of probiotic organisms have been used to convert IG to IA due to β-glucosidase activity they possess (Chien et al., 2006; Otieno, Ashton, & Shah, 2007; Shah, 2006; Tsangalis et al., 2002; Wei et al., 2007). However, the biotransformation rate of IG to IA by probiotic bacteria in general was considerably low in fermented soymilk (Chien et al., 2006; Tsangalis et al., 2002). To enhance the biotransformation level, soymilk (SM) could be supplemented with skim milk powder (SMP). Milk is considered a poor medium for the growth of microorganisms due to lack of amino acids such as lysine, which are found abundantly (5.3%) in SPI after the hydrolysis by probiotic organisms during the incubation (Hofman & Thonart, 2001; Nutrition Data, 2007; Sugano, 2005). On the other hand, milk could provide a source of lactose for probiotic microorganisms which are known to grow in milk based medium (Sugano, 2005). Lactose is also considered a bifidogenic factor which stimulates the growth and metabolism of lactobacilli and bifidobacteria (Dubey & Mistry, 1996; Kontula et al., 1999). If pure lactose is used instead of SMP, it is assumed that the biotransformation of isoflavone glycosides to aglycones and the growth of the probiotic would be still enhanced but the enhancing effect may be not as good as SMP. The supplementation with SMP did not only enhance the biotransformation but also increased the market value of the fermented products. Consequently, probiotic organisms are expected to grow better in SM supplemented with SMP than SM alone, and the biotransformation of IG to IA is also expected to be enhanced. The combination of SPI and skim milk could provide an excellent and complete medium for the growth of probiotic organisms. Compared to the lactulose supplementation (chapter 4.0), the supplementation with SMP would enhance the commercial values of the fermented products as well. However, to date, there is no report about the fermentation of SM supplementation with SMP by the predominant probiotic *Lactobacillus* group. Therefore, the objectives of this study were (i) to investigate lactose utilisation by *Lactobacillus* and their survival, and (ii) the biotransformation of IG to IA in SM prepared from SPI supplemented with SMP.

5.1.2 Materials and methods

5.1.2.1 Isoflavone compounds and other chemicals

Isoflavone compounds and other chemicals are described as section 4.1.2.1. Skim milk powder was from Murray Goulburn Co-Operative Company (Brunswick, Vic, Australia).

5.1.2.2 Cultures and fermentation of soymilk supplemented with skim milk powder (SSM), soymilk (SM) and reconstituted skim milk (RSM) by Lactobacillus

Lactobacillus acidophilus 4461, *L. acidophilus* 4962, *L. casei* 290 and *L. casei* 2607 were obtained from the Victoria University Culture Collection (Werribee, Vic, Australia). The purity of cultures was checked and the probiotic organisms were stored at -80 ^{o}C in 40% (v/v) sterile glycerol. They were individually activated in de Mann Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK) (pH adjusted to 6.7 using 5M NaOH) successively twice at 37 ^{o}C for 20 h. The third transfer was carried out separately in (i) SSM prepared from 4% (w/v) SPI supplemented with 12% (w/v) SMP (SSM), (ii) SM prepared from 4% SPI (w/v), and (iii) RSM prepared from 12% (w/v) SMP. One litter of sterile SSM, SM and RSM were individually inoculated with 1% (v/v) of the organisms from the third transfer and incubated anaerobically at 37 ^{o}C for 24 h. One hundred milliliter aliquots were withdrawn aseptically at 0, 6, 12, 18 and 24 h of incubation for enumeration of viable probiotic bacteria, measurement of pH and determination of lactose, and the remainder of the samples was freeze-dried using a Dynavac freeze-dryer (model FD 300; Rowville, Vic, Australia) for quantification of isoflavone.

5.1.2.3 Determination of pH

Determination of pH is described as section 4.1.2.4

5.1.2.4 Determination of lactose contents

Quantification of lactose was based on Chavez-Servin et al. (2004) with some modifications. Briefly, one milliliter of SSM or RSM was added to 10 mL of aqueous ethanol (50:50, v/v) in a tube and placed in a 60 ^{o}C water bath (model NB 6T-10935, Thermoline Australia, Scientific Equipments, Smithfield, NSW, Australia) until completely dissolved. To this, 250 µL of each of Carrez I and Carrez II solutions and 5 mL of acetonitrile were added. The solution was made up to 50 mL using aqueous ethanol (50:50, v/v), then filtered through Advance No 1 filter paper, a C18 Sep-pak Plus cartridge (Waters, Milford, MA, USA) and a 0.45 µm nylon filter (Phenomenex, Lane Cove, NSW, Australia) and then injected into the HPLC system. Instrument and HPLC conditions included an Alltech Alltima (Deerfield, IL, USA) Prevail-Carbohydrate ES (4.6 x 250) mm column with a 5 µm particle size and a Hewlett Packard 1100 series HPLC (Agilent Technologies, Forest Hill, Vic, Australia) with an auto sampler, a quaternary pump, an Alltech light-scattering detector Varex MK III ELSD, a vacuum degasser and a thermostatically controlled column compartment. The injection volume was 20 µL. Mobile phase for isocratic HPLC was acetonitrile: water (70:30, v/v). Flow rate was at 0.8 mL/min. Standard solutions for calibration curve were based on five lactose working solutions prepared by diluting pure lactose with methanol (50%, v/v) at various concentrations between 50 µg/mL to 500 µg/mL.

5.1.2.5 Enumeration of viable micro-organisms

Enumeration of viable microorganisms is described as section 4.1.2.3

5.1.2.6 Determination of isoflavone contents

Determination of isoflavone contents is described as section 3.2.5, 3.2.6 and 4.1.2.6

5.1.2.7 Statistical analysis of data

Statistical analysis of data is described as section 4.1.2.7

5.1.3 Results and Discussion

5.1.3.1 Lactose utilisation and pH changes in RSM and SSM during fermentation by Lactobacillus

The initial lactose content in SSM and RSM was 52.85 and 55.28 mg/mL, respectively. There was no lactose present in SM (Nutrition Data, 2007). The pH of SM decreased slightly from 6.80 to 6.29 during 24 h fermentation by *Lactobacillus* (Figures. 5.1, 5.2, 5.3, and 5.4). On the other hand, the pH of SSM and RSM decreased to 4.07 to 4.32 and 4.59 to 4.96, respectively. The pH values of SSM were significantly lower ($P < 0.05$) and the drop was much faster compared to those in SM. The reason may due to the presence of lactose in SSM and low level of simple sugars in SM (Nutrition Data, 2007). Vedamuthu (2006) reported that the products of lactose fermentation by *Lactobacillus* included acetic and lactic acids, which lowered the pH of the medium. This result was in agreement with Tsangalis & Shah (2004), who reported that the pH values decreased from 6.5 in SM to 6.0 in SM supplemented with glucose by *B. longum* 1941. In our study, the drop in the pH was faster and the pH values were lower in SSM than in RSM by all the four probiotic organisms during the entire incubation. This is possibly due to lower buffering capacity of soy protein compared to milk proteins or the higher solid content in SSM (Farnworth et al., 2007). Therefore, the supplementation with SMP appears to have played a key role in reduction of the pH in SSM.

Lactose utilisation by *Lactobacillus* in SSM was higher than that in RSM during the entire incubation. However, only *L. acidophilus* 4461 and *L. acidophilus* 4962 utilised a significantly higher ($P < 0.05$) level of lactose in SSM than that in RSM (Figures 5.1 and 5.2). *Lactobacillus acidophilus* 4461 utilised the highest level of lactose at 18.15 and 16.06 mg/mL in SSM and RSM, and decreased the pH to lowest level, at 4.07 and 4.59, respectively, after 24 h of incubation (Figure 5.1). *Lactobacillus casei* 2607 utilised the lowest level of lactose at 14.12 mg/mL in RSM after 24 h of incubation and the pH of the medium remained at 4.96 (Figure 5.4). All the four probiotic organisms utilised up to 2.19 mg/mL higher amount of lactose in SSM than RSM during the incubation. Therefore, our results suggest that the presence of SPI in SSM enhanced the lactose utilisation by *Lactobacillus*. Poch & Bezkorovainy (1988) reported the presence

of some essential amino acids, including tryptophan, isoleucine, cysteine and tyrosine, which are all found in SPI, to stimulate the growth of probiotic organisms. Hence, these amino acids may have enhanced the lactose metabolism by probiotic organisms. However, the effect was not as obvious by *L. casei* 290 (Figure 5.3) and *L. casei* 2607 (Figure 5.4) as was with by *L. acidophilus* 4461 (Figure 5.1) and *L. acidophilus* 4962 (Figure 5.2). On the other hand, the decrease in pH was found to be important for lactose utilisation by probiotic organisms. *Lactobacillus acidophilus* 4461 used the lactose highest level of lactose in both SSM and RSM, and as a result, pH values decreased to lowest levels (Figure 5.1).

5.1.3.2 Survival of probiotic organisms in SSM, RSM and SM

Figure 5.5 shows the viable microbial counts in log CFU/mL in SSM, RSM and SM during fermentation by *Lactobacillus*. In general, *Lactobacillus* showed the least survival in SM and the highest survival in RSM during the incubation period of 24 h. It has been reported that SM did not support the growth of probiotic organisms as much as did RSM (Kamaly, 1997). The survival of the probiotic organisms in SSM was significantly higher ($P < 0.05$) than that in SM after 12 h of incubation by all the four probiotic organisms. The viable counts of *Lactobacillus* in SSM were 0.36 to 0.98 log CFU/mL higher than those in SM. Therefore, it appeared that the supplementation with SMP to SM enhanced the viable counts of probiotic organisms. However, the presence of SPI in SSM did not support the growth of probiotic organisms. The survival of the probiotic organisms in SSM was lower than that in RSM. The pH in SSM may have played a key role in decreasing the survival of the probiotic organisms as the pH in SSM was lower than that of RSM during the incubation (Figure 5.5). Gomes, Malcata, & Klaver (1998) also reported that *L. acidophilus* Ki showed lower viable counts in milk supplemented with milk hydrolyzate, compared to those in milk alone. On the other hand, the viable counts of *Lactobacillus* in SSM and RSM decreased after 18 h of incubation while remained stable in SM as the pH in the later was still within the favourable range for the growth of probiotic organisms (Shah, 2006).

5.1.3.3 Biotransformation of IG to IA in SM and SSM by Lactobacillus

The moisture content of freeze-dried samples ranged from 1.95 to 2.02%. There were no significant ($P > 0.05$) differences in moisture contents of the freeze-dried samples. Therefore, we assumed that there was no effect of the moisture content on the estimation of isoflavones. The HPLC chromatogram and the retention time of 14 standard isoflavone compounds and the internal standard are shown in Figure 3.1. Tables 5.1 – 5.4 present the transformation of IG to IA in SM and SSM by *Lactobacillus*. There were only 8 isoflavone compounds detected in the SM or SSM at 0 h (Table 5.1). The total isoflavone content in SM and SSM at 0 h was 153.31 and 35.37 mg/ 100 g of freeze-dried sample, respectively. The lower initial level of the isoflavone compounds in SSM was due to the supplementation with SMP. The level of the isoflavones in SM prepared from SPI (153.31 mg/100 g freeze-dried sample) was less than that in soy flour (188-276 mg/100 g sample) as reported by King and Bignell (2000). Wang & Murphy (1996) reported that the mild alkali extraction in the production of SPI causes isoflavones losses of up to 53%. Table 5.1 presents the biotransformation of IG to IA by *L. acidophilus* 4461 in SSM and SM. Supplementation with SMP appeared to enhance the biotransformation of IG to IA during incubation. At 12 h of incubation, 76.0% of IG in SSM was hydrolysed compared to 65.4% in SM. At the end of the fermentation, 83.5% of IG was hydrolysed and IA fraction increased from 3.6% to 74.1% of total isoflavones available in SSM. On the other hand, the fermentation of acetyl daidzin appeared to be lower than that for daidzin during the incubation. At 6 h of incubation, the amount of daidzin and acetyl daidzin hydrolysed in SSM was 3.37 and 0.22 mg/100 g of freeze- dried sample, respectively (Table 5.1).

The supplementation with SMP appeared to have the greatest stimulating effect on the biotransformation of IG to IA by *L. acidophilus* 4962 (Table 5.2). Most of IG was hydrolysed completely in SSM while they were still present in SM at 24 h of incubation. The biotransformation level was enhanced extensively from 66.1% in SM to 85.1% in SSM. After the fermentation, IG comprised only a minor fraction (23.2%) of total isoflavones in SSM.

Table 5.3 shows the biotransformation of IG to IA by *L. casei* 290. As shown in the table, the supplementation with SMP enhanced the biotransformation of IG effectively during the first 6 h of incubation. At 6 h of incubation, only 37.0% of IG in SM were hydrolysed compared to 72.7% in SSM. At the end of the incubation, 67.5% of IG was transformed in SM, compared to 81.4% in SSM. Similarly, the supplementation with SMP in SSM increased the biotransformation level from 67.3 to 81.7% at 24 h of incubation (Table 5.4). The biotransformation in both SSM and SM occurred rapidly in the first 12 h of incubation by all the four probiotic organisms. For the next 12 h of incubation, the level of biotransformation increased slowly. There was no significant difference ($P > 0.05$) between the IA produced at 18 and 24 h of incubation in both SM and SSM. The supplementation with SMP showed an increase in biotransformation of IG to IA by all the four probiotic organisms.

The enhanced biotransformation in SSM is possibly due to the presence of lactose in SMP. *Lactobacillus* must produce β-galactosidase in order to breakdown lactose in SSM into galactose and glucose. β-Galactosidase was found to hydrolyse IG to IA (referring to Chapter 3.0). Therefore, lactose may have played a key role in enhancing the biotransformation level by possibly increasing the production of β-galactosidase by *Lactobacillus*. Low pH condition in SSM may have also contributed to the increase in the biotransformation level. Delmonte et al. (2006) reported that some IG was partly hydrolysed to IA in a low pH condition.

5.1.4 Conclusion

Supplementation with SMP significantly ($P < 0.05$) stimulated the growth of *Lactobacillus* by providing lactose and others nutrients. Consequently, the biotransformation of IG in SSM was significantly increased by 13.9 to 19.0%, after 24 h of incubation. Supplementation with SMP also played a key role in decreasing the pH of SSM. The presence of SPI stimulated the lactose utilisation in SSM, but the effect varied with lactobacilli. The low pH condition in SSM decreased the viable counts of *Lactobacillus*.

Table 5.1 Biotransformation of IG to IA in SSM and SM by *L. acidophilus* 4461 at 37 ^{o}C

Isoflavone (mg/100 g of freeze-dried sample)	SSM					SM				
	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
Daidzin	3.37 ± 0.15	ND	ND	ND	ND	14.03 ± 0.70^{a}	8.23 ± 0.62^{b}	2.08 ± 0.21^{c}	ND	ND
Glycitin	1.19 ± 0.05	ND	ND	ND	ND	6.13 ± 0.10	ND	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	4.81 ± 0.25^{a}	0.50 ± 0.04^{b}	0.46 ± 0.09^{b}	0.45 ± 0.05^{b}	ND	24.49 ± 1.69^{a}	12.05 ± 1.04^{b}	4.23± 0.35^{c}	4.26 ± 0.31^{c}	4.19 ± 0.28^{c}
Malonyl glycitin	1.06 ± 0.05^{a}	0.73 ± 0.05^{b}	0.85 ± 0.07^{b}	0.87 ± 0.09^{ab}	0.83 ± 0.07^{b}	3.02 ± 0.25^{a}	3.01 ± 0.21^{a}	ND	ND	ND
Malonyl genistin	16.13 ± 0.72^{a}	8.79 ± 0.75^{b}	5.67 ± 0.32^{c}	5.01 ± 0.45^{c}	4.41 ± 0.41^{c}	67.23 ± 2.02^{a}	28.80 ± 1.54^{b}	27.58 ± 1.65^{b}	27.71 ± 1.32^{bc}	26.02 ± 1.04^{c}
Acetyl daidzin	1.52 ± 0.08^{a}	1.30 ± 0.10^{b}	ND	ND	ND	6.41 ± 0.19^{a}	6.34 ± 0.40^{a}	3.07 ± 0.15^{b}	3.00 ± 0.24^{b}	3.01± 0.17^{b}
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acetyl genistin	6.03 ± 0.29^{a}	2.29 ± 0.14^{b}	1.21 ± 0.11^{c}	ND	ND	27.50 ± 1.63^{a}	14.70 ± 1.07^{b}	14.55 ± 1.85^{b}	14.82 ± 0.98^{b}	14.13 ± 0.88^{b}
Total IG	***34.11 ± 1.59^{a}***	***13.61 ± 0.80^{b}***	***8.19 ± 0.23^{c}***	***6.33 ± 0.49^{c}***	***5.63 ± 0.51^{c}***	***148.81 ± 2.94^{a}***	***73.13 ± 0.70^{b}***	***51.51 ± 4.21^{c}***	***49.79 ± 2.23^{c}***	***47.35 ± 1.81^{c}***
Daidzein	ND	3.67 ± 0.21^{a}	3.97 ± 0.13^{a}	4.14 ± 0.17^{a}	4.69 ± 0.25^{a}	ND	8.42 ± 0.71^{a}	17.89 ± 1.72^{b}	18.70 ± 1.24^{b}	19.03 ± 1.55^{b}
Glycitein	ND	0.51 ± 0.04^{a}	0.53 ± 0.24^{a}	0.58 ± 0.06^{a}	0.65 ± 0.04^{a}	ND	2.75 ± 0.15^{a}	3.71 ± 1.54^{a}	3.87 ± 0.25^{a}	3.79 ± 0.21^{a}
Genistein	1.26 ± 0.08^{a}	6.18 ± 0.42^{b}	8.34 ± 0.55^{c}	9.41 ± 0.73^{c}	10.81 ± 0.92^{c}	4.50 ± 0.32^{a}	30.86 ± 2.10^{b}	33.09 ± 1.85^{b}	33.28 ± 2.14^{b}	37.91± 2.17^{c}
Total IA	***1.26 ± 0.08^{a}***	***10.36 ± 0.67^{b}***	***12.84 ± 0.92^{bc}***	***14.13 ± 0.96^{cd}***	***16.15 ± 1.21^{d}***	***4.50 ± 0.32^{a}***	***42.03 ± 2.66^{b}***	***54.69 ± 2.03^{c}***	***55.85 ± 3.63^{c}***	***60.73 ± 3.51^{c}***
IG hydrolysed (%)	***0.0***	***60.1***	***76.0***	***81.4***	***83.5***	***0.0***	***50.9***	***65.4***	***66.5***	***68.2***

Results expressed as mean ± standard error (n = 3). Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. ND: Not detected (the isoflavone content which was in 1 g freeze dried sample used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method). SSM: soymilk supplemented with skim milk powder. SM: soymilk. IG: isoflavone glycosides. IA: isoflavone aglycones

Table 5.2 Biotransformation of IG to IA in SSM and SM by *L. acidophilus* 4962 at 37 ^{o}C

Isoflavone (mg/100 g of freeze-dried sample)	SSM					SM				
	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
Daidzin	3.37 ± 0.15^{a}	1.15 ± 0.12^{b}	0.88 ± 0.05^{b}	ND	ND	14.03 ± 0.70^{a}	13.95 ± 1.03^{a}	4.01 ± 0.48^{b}	3.72 ± 0.30^{b}	3.64 ± 0.32^{b}
Glycitin	1.19 ± 0.05	ND	ND	ND	ND	6.13 ± 0.10^{a}	5.50 ± 0.45^{a}	3.02 ± 0.20^{b}	2.39 ± 0.25^{b}	2.50 ± 0.24^{b}
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	4.81 ± 0.25^{a}	0.55 ± 0.07^{b}	0.48 ± 0.03^{b}	ND	ND	24.49 ± 1.69^{a}	24.03 ± 1.52^{a}	13.72 ± 1.08^{b}	8.53 ± 0.92^{c}	7.56 ± 0.54^{c}
Malonyl glycitin	1.06 ± 0.05^{a}	0.95 ± 0.06^{a}	0.90 ± 0.07^{a}	0.70 ± 0.04^{b}	ND	3.02 ± 0.25^{a}	2.32 ± 0.20^{b}	ND	ND	ND
Malonyl genistin	16.13 ± 0.72^{a}	6.08 ± 0.21^{b}	5.45 ± 0.42^{bc}	4.47 ± 0.34^{c}	5.08 ± 0.42^{c}	67.23 ± 2.02^{a}	27.12 ± 1.41^{b}	25.03 ± 1.08^{b}	25.76 ± 1.25^{b}	23.73 ± 1.87^{b}
Acetyl daidzin	1.52 ± 0.08^{a}	ND	ND	ND	ND	6.41 ± 0.19^{a}	5.93 ± 0.65^{a}	4.82 ± 0.31^{ab}	3.91 ± 0.41^{b}	3.75 ± 0.25^{b}
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acetyl genistin	6.03 ± 0.29^{a}	2.63 ± 0.14^{b}	1.54 ± 0.15^{c}	ND	ND	27.50 ± 1.63^{a}	13.85 ± 0.86^{b}	13.63 ± 1.03^{b}	13.60 ± 1.07^{b}	9.27 ± 0.75^{c}
Total IG	***34.11 ± 1.59^{a}***	***11.37 ± 0.08^{b}***	***9.25 ± 0.72^{b}***	***5.17 ± 0.30^{c}***	***5.08 ± 0.42^{c}***	***148.81 ± 2.94^{a}***	***92.70 ± 4.40^{b}***	***64.23 ± 4.18^{c}***	***57.91 ± 1.76^{cd}***	***50.45 ± 1.99^{d}***
Daidzein	ND	3.97 ± 0.25^{a}	3.99 ± 0.32^{a}	4.77 ± 0.32^{ab}	4.98 ± 0.32^{b}	ND	1.04 ± 0.21^{a}	13.53 ± 1.24^{b}	14.39 ± 1.08^{b}	15.21 ± 1.32^{b}
Glycitein	ND	0.45 ± 0.02^{a}	0.52 ± 0.05^{a}	0.62 ± 0.04^{a}	0.89 ± 0.05^{a}	ND	0.90 ± 0.32^{a}	3.45 ± 0.25^{b}	3.48 ± 0.25^{b}	3.53 ± 0.11^{b}
Genistein	1.26 ± 0.08^{a}	8.45 ± 0.74^{b}	8.12 ± 0.71^{b}	10.03 ± 0.75^{bc}	10.92 ± 0.87^{c}	4.50 ± 0.32^{a}	32.23 ± 1.98^{b}	33.99 ± 3.04^{b}	33.50 ± 1.52^{b}	35.37 ± 2.35^{b}
Total of aglycones	***1.26 ± 0.08^{a}***	***12.87 ± 0.97^{b}***	***12.63 ± 0.34^{b}***	***15.12 ± 1.11^{c}***	***16.80 ± 0.50^{c}***	***4.50 ± 0.32^{a}***	***34.17 ± 2.51^{b}***	***50.97 ± 1.55^{c}***	***51.37 ± 2.35^{c}***	***54.11 ± 1.14^{c}***
IG hydrolysed (%)	***0.0***	***66.7***	***72.9***	***84.8***	***85.1***	***0.0***	***37.7***	***56.8***	***61.1***	***66.1***

Results expressed as mean ± standard error (n = 3). Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. ND: Not detected (the isoflavone content which was in 1 g freeze dried sample used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method). SSM: soymilk supplemented with skim milk powder. SM: soymilk. IG: isoflavone glycosides. IA: isoflavone aglycones

Table 5.3 Biotransformation of IG to IA in SSM and SM by *L. casei* 290 at 37 ^{o}C

Isoflavone	SSM					SM				
(mg/100 g of freeze-dried sample)	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
Daidzin	3.37 ± 0.15	ND	ND	ND	ND	14.03 ± 0.70^{a}	12.65 ± 1.07^{a}	5.35 ± 0.53^{b}	4.32 ± 0.25^{bc}	3.15 ± 0.25^{c}
Glycitin	1.19 ± 0.05	ND	ND	ND	ND	6.13 ± 0.10^{a}	5.21± 0.32^{b}	3.82 ± 0.20^{c}	3.08 ± 0.14^{d}	2.85 ± 0.15^{d}
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	4.81 ± 0.25	ND	ND	ND	ND	24.49 ± 1.69^{a}	20.83 ± 1.54^{b}	5.04 ± 0.43^{c}	3.81 ± 0.25^{cd}	2.69 ± 0.12^{d}
Malonyl glycitin	1.06 ± 0.05^{a}	ND	ND	ND	ND	3.02 ± 0.25^{a}	2.63± 0.12^{b}	ND	ND	ND
Malonyl genistin	16.13 ± 0.72^{a}	6.45 ± 0.42^{b}	6.22 ± 0.41^{b}	6.67 ± 0.51^{b}	6.34 ± 0.37^{b}	67.23 ± 2.02^{a}	30.33 ± 2.61^{b}	27.17 ± 2.20^{b}	25.91 ± 2.01^{b}	24.74 ± 1.85^{b}
Acetyl daidzin	1.52 ± 0.08^{a}	ND	ND	ND	ND	6.41 ± 0.19^{a}	6.26 ± 0.52^{a}	5.84 ± 0.41^{a}	4.02 ± 0.29^{b}	3.56 ± 0.24^{b}
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acetyl genistin	6.03 ± 0.29^{a}	2.88 ± 0.17^{b}	2.62 ± 0.14^{b}	ND	ND	27.50 ± 1.63^{a}	15.83 ± 1.09^{b}	12.25 ± 1.04^{c}	12.42 ± 0.99^{bc}	11.37 ± 0.87^{c}
Total IG	***34.11 ± 1.59^{a}***	***9.33 ± 0.59^{b}***	***8.84 ± 0.27^{bc}***	***6.67 ± 0.51^{cd}***	***6.34 ± 0.37^{d}***	***148.81 ± 2.94^{a}***	***93.74 ± 3.81^{b}***	***59.47 ± 1.91^{c}***	***53.56 ± 1.37^{c}***	***48.36 ± 1.26^{d}***
Daidzein	ND	3.62 ± 0.15^{a}	4.20 ± 0.23^{ab}	4.40 ± 0.32^{b}	4.24 ± 0.28^{ab}	ND	2.48 ± 0.36^{a}	17.19 ± 1.04^{b}	18.36 ± 1.24^{b}	19.06 ± 1.24^{b}
Glycitein	ND	1.00 ± 0.14^{a}	1.03 ± 0.08^{a}	1.10 ± 0.09^{a}	1.10 ± 0.08^{a}	ND	0.62 ± 0.15^{a}	2.87 ± 0.24^{a}	2.98 ± 0.25^{a}	3.45 ± 0.21^{a}
Genistein	1.26 ± 0.08^{a}	8.63 ± 0.74^{b}	9.75 ± 0.99^{b}	9.73 ± 0.54^{b}	10.24 ± 1.03^{b}	4.5 ± 0.32^{a}	29.62 ± 1.98^{b}	33.82 ± 2.14^{b}	34.47 ± 2.41^{b}	34.95 ± 2.24^{b}
Total IA	***1.26 ± 0.08^{a}***	***13.25 ± 0.45^{b}***	***14.98 ± 0.84^{b}***	***15.23 ± 0.95^{b}***	***15.58 ± 1.23^{b}***	***4.5± 0.32^{a}***	***32.72 ± 2.49^{b}***	***53.88 ± 2.94^{c}***	***55.81 ± 1.42^{c}***	***57.46 ±1.21^{c}***
IG hydrolysed (%)	***0.0***	***72.7***	***74.1***	***80.5***	***81.4***	***0.0***	***37.0***	***60.0***	***64.0***	***67.5***

Results expressed as mean ± standard error (n = 3). Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. ND: Not detected (the isoflavone content which was in 1 g freeze dried sample used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method). SSM: soymilk supplemented with skim milk powder. SM: soymilk. IG: isoflavone glycosides. IA: isoflavone aglycones

Table 5.4 Biotransformation of IG to IA in SSM and SM by *L. casei* 2607 at 37 ^{o}C

Isoflavone (mg/100 g of freeze-dried sample)	SSM					SM				
	0 h	6 h	12 h	18 h	24 h	0 h	6 h	12 h	18 h	24 h
Daidzin	3.37 ± 0.15^{a}	2.13 ± 0.16^{b}	ND	ND	ND	14.03 ± 0.70^{a}	13.59 ± 1.09^{a}	6.60 ± 0.78^{c}	4.21± 0.25^{d}	3.15 ± 0.21^{d}
Glycitin	1.19 ± 0.05	ND	ND	ND	ND	6.13 ± 0.10^{a}	5.32 ± 0.35^{b}	4.12 ± 0.19^{c}	3.01± 0.12^{d}	2.21± 0.12^{c}
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	4.81 ± 0.25^{a}	3.37 ± 0.21^{b}	1.03 ± 0.15^{c}	ND	ND	24.49 ± 1.69^{a}	23.21 ± 1.15^{a}	5.35 ± 0.39^{b}	5.04 ± 0.25^{b}	4.78 ± 0.19^{b}
Malonyl glycitin	1.06 ± 0.05^{a}	1.02 ± 0.08^{a}	1.03 ± 0.12^{a}	ND	ND	3.02 ± 0.25^{a}	3.00 ± 0.25^{a}	2.85 ± 0.20^{a}	2.97 ± 0.36^{a}	2.93 ± 0.30^{a}
Malonyl genistin	16.13 ± 0.72^{a}	7.80 ± 0.52^{b}	6.22 ± 0.42^{b}	6.20 ± 0.45^{b}	6.24 ± 0.51^{b}	67.23 ± 2.02^{a}	33.49 ± 2.50^{b}	28.70 ± 1.68^{bc}	23.62± 1.27^{cd}	22.15 ± 1.52^{d}
Acetyl daidzin	1.52 ± 0.08^{a}	ND	ND	ND	ND	6.41 ± 0.19^{a}	6.23 ± 0.42^{a}	5.14 ± 0.41^{b}	5.00 ± 0.35^{b}	4.55 ± 0.29^{b}
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acetyl genistin	6.03 ± 0.29^{a}	3.60 ± 0.26^{c}	2.94 ± 0.19^{c}	1.90 ± 0.15^{d}	ND	27.50 ± 1.63^{a}	16.46 ± 1.19^{b}	15.57± 1.23^{b}	10.53 ± 0.98^{c}	8.83 ± 0.79^{c}
Total of IG	***34.11 ± 1.59^{a}***	***17.92 ± 1.23^{b}***	***11.23 ± 1.00^{c}***	***8.10 ± 0.60^{cd}***	***6.24 ± 0.51^{d}***	***148.81 ± 2.94^{a}***	***101.30 ± 3.46^{b}***	***68.33 ± 4.69^{c}***	***54.38 ± 1.54^{d}***	***48.60 ± 0.26^{d}***
Daidzein	ND	1.36 ± 0.12^{a}	4.07 ± 0.25^{b}	4.16 ± 0.28^{b}	4.26 ± 0.26^{b}	ND	0.69 ± 0.05^{a}	15.49 ± 1.24^{b}	16.37 ± 1.28^{b}	17.59 ± 1.08^{b}
Glycitein	ND	0.70 ± 0.05^{a}	0.76 ± 0.05^{a}	0.88 ± 0.07^{ab}	0.97 ± 0.08^{b}	ND	0.55 ± 0.24^{a}	1.03 ± 0.19^{a}	2.25 ± 0.19^{a}	2.62 ± 0.25^{a}
Genistein	1.26 ± 0.08^{a}	6.09 ± 0.07^{b}	8.68 ± 0.48^{c}	9.13 ± 0.56^{cd}	10.44 ± 1.03^{d}	4.5 ± 0.32^{a}	27.85 ± 1.57^{b}	32.38 ± 2.12^{bc}	36.18 ± 1.95^{c}	37.36 ± 1.69^{c}
Total IA	***1.26 ± 0.08^{a}***	***8.15 ± 0.24^{b}***	***13.51 ± 0.78^{c}***	***14.17 ± 0.91^{c}***	***15.67 ± 1.37^{c}***	***4.5± 0.32^{a}***	***29.09 ± 1.28^{b}***	***48.90 ± 1.07^{c}***	***54.80 ± 0.86^{d}***	***57.37 ± 3.02^{d}***
IG hydrolysed (%)	***0.0***	***47.5***	***67.1***	***76.3***	***81.7***	***0.0***	***31.9***	***54.1***	***63.5***	***67.3***

Results expressed as mean ± standard error (n=3). Mean values in the same row for a particular medium with the same lowercase superscripts are not significantly different (P>0.05). IG: Isoflavone glycosides. ND: Not detected (the isoflavone content which was in 1 g freeze dried sample used to extract isoflavones with a sample injection volume of 20 µL was lower than the detection limit of the method). SSM: soymilk supplemented with skim milk powder. SM: soymilk. IG: isoflavone glycosides. IA: isoflavone aglycones

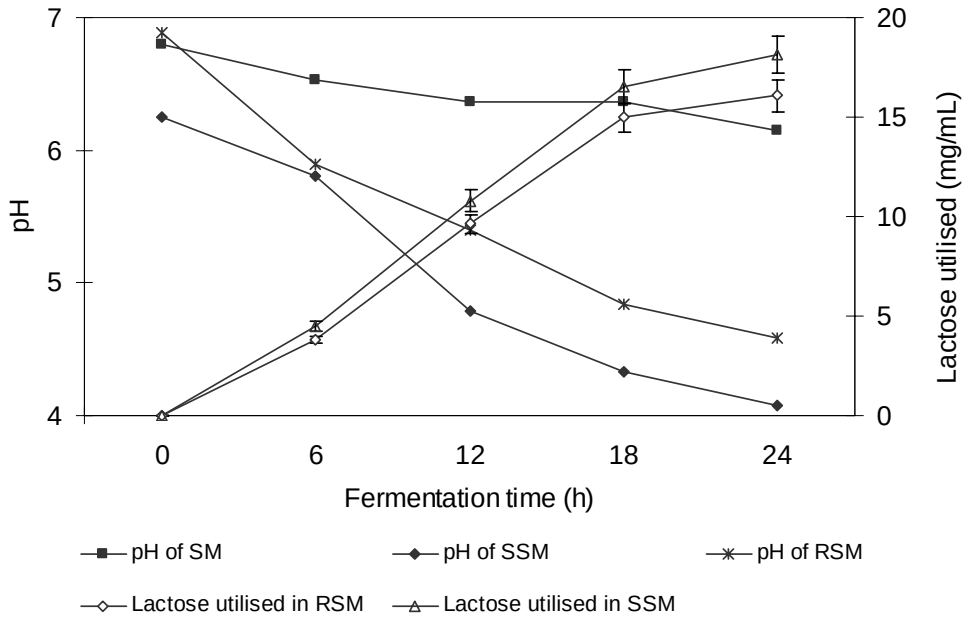


Figure 5.1 pH values and lactose utilisation (mg/mL) of RSM, SM and SSM fermented by *L. acidophilus* 4461 for 24 h at 37 ^{o}C

Results are expressed as mean ± standard error (n = 3)

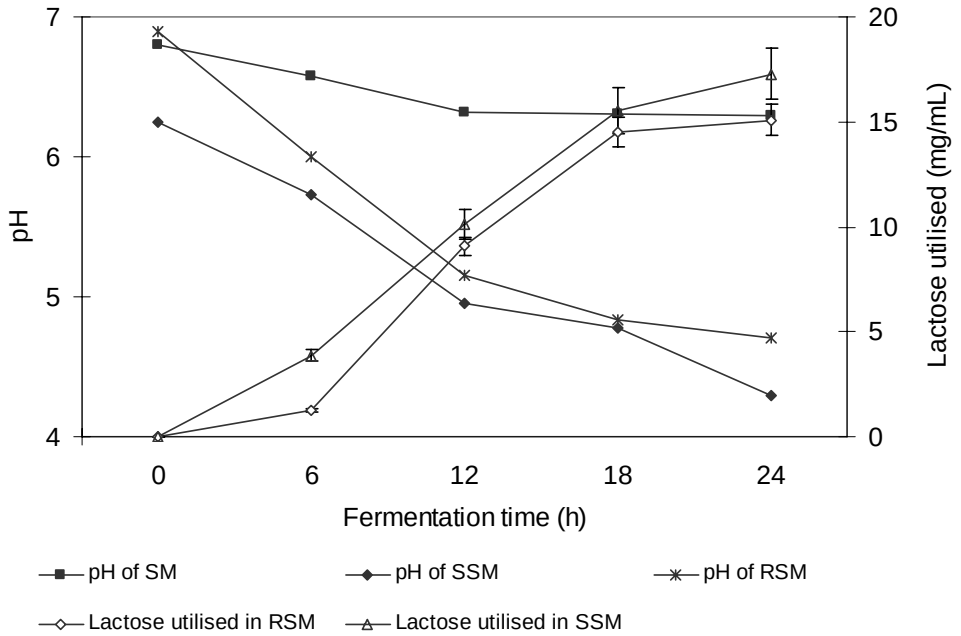


Figure 5.2 pH values and lactose utilisation (mg/mL) of RSM, SM and SSM fermented by *L. acidophilus* 4962 for 24 h at 37 ^{o}C

Results are expressed as mean ± standard error (n = 3)

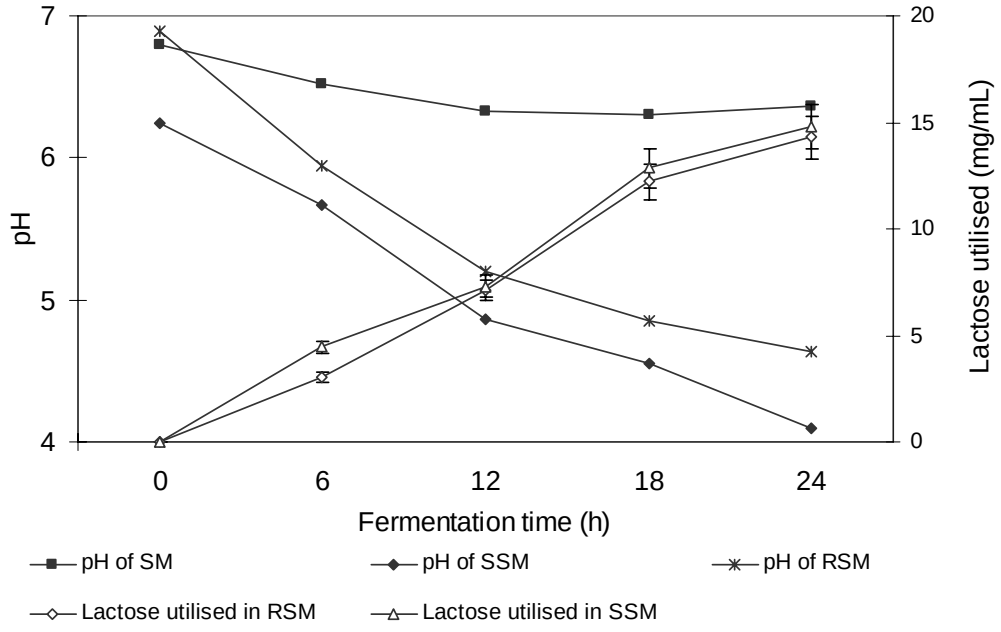


Figure 5.3 pH values and lactose utilisation (mg/mL) of RSM, SM and SSM fermented by *L. casei* 290 for 24 h at 37 ^{o}C

Results are expressed as mean ± standard error (n = 3)

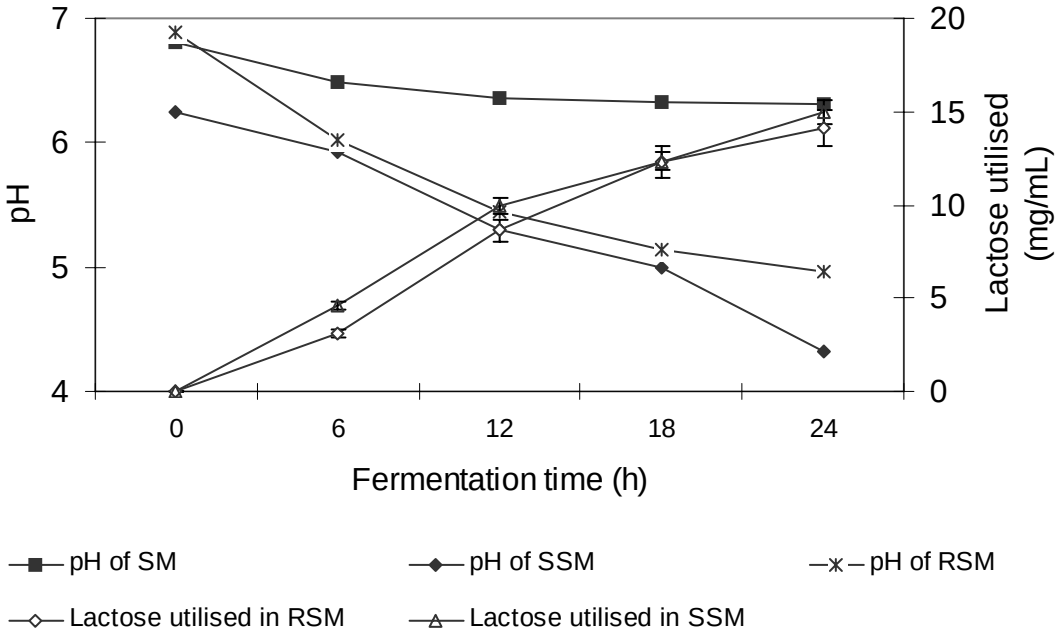


Figure 5.4 pH values and lactose utilisation (mg/mL) of RSM, SM and SSM fermented by *L. casei* 2607 for 24 h at 37 ^{o}C

Results are expressed as mean ± standard error (n = 3)

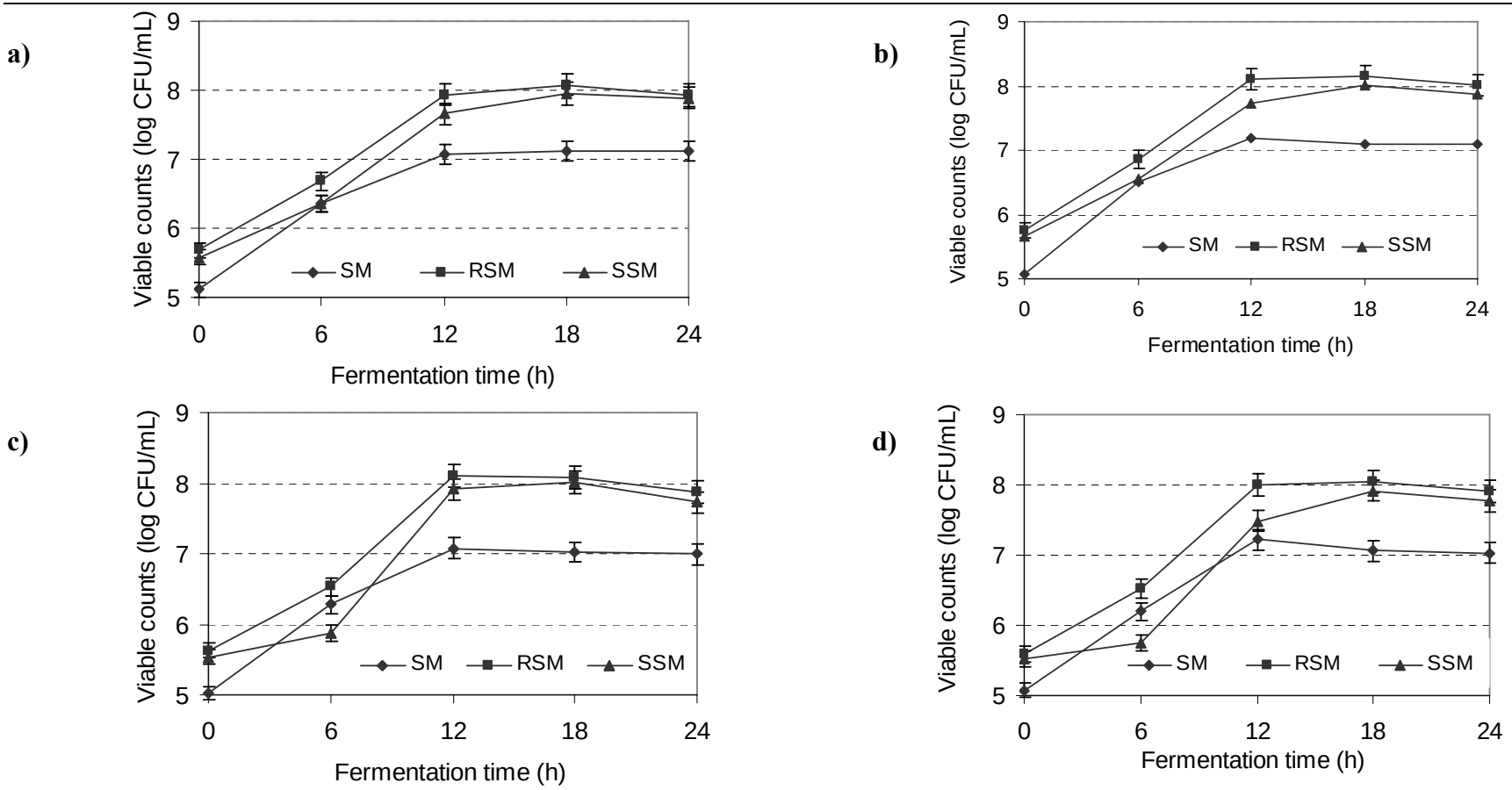


Figure 5.5 Viable microbial counts (log CFU/mL) of *Lactobacillus* in RSM, SM and SSM fermented for 24 h at 37 ^{o}C

Results are expressed as mean ± standard error (n = 3)

a) Viable microbial counts (log CFU/mL) of *L. acidophilus* 4461 b) Viable microbial counts (log CFU/mL) of *L. acidophilus* 4962

c) Viable microbial counts (log CFU/mL) of *L. casei* 290 d) Viable microbial counts (log CFU/mL) of *L. casei* 2607

5.2 Biotransformation of isoflavone glycosides by *Bifidobacterium* in soymilk supplemented with skim milk powder

5.2.1 Introduction

Isoflavone aglycones (IA) are absorbed directly through the gut wall, while isoflavone glycosides (IG) are very poorly absorbed from the gut due to their higher hydrophilicity and larger molecular weight. The IA are absorbed faster and in greater amounts than their glycosides counterpart (IG) (Izumi et al., 2000). It is generally thought that IG are converted to their corresponding aglycones by gut microflora or gut glucosidases and then absorbed from the small intestine (Izumi et al., 2000). Several groups of gut bacteria such as *Bifidobacterium*, due to β-glucosidase activity, are able to hydrolyse IG to aglycones (Hughes et al., 2003; Izumi et al., 2000; Otieno et al., 2005; Tsangalis et al., 2002). However, only about 30-50% of individuals in Western population are able to metabolize IG to aglycones and aglycones to equol, in the intestinal tract (Frankenfeld et al., 2005; Higdon, 2006). In addition, aglycones have been reported to be more stable than IG during the storage at different temperatures (Otieno et al., 2006b). Consequently, providing food products with aglycones would be considered as a novel trend for the food industry.

In the last few years, several scientists have reported the transformation of IG to aglycones by bifidobacteria and lactobacilli. These organisms are classified as probiotics, which are defined as live microbial supplements that provide beneficial effect to the host by improving its intestinal microbial balance (Shah, 2006). Tsangalis et al. (2002) studied the enzymic transformation of isoflavone phytoestrogens in soymilk by β-glucosidase-producing bifidobacteria. Otieno et al. (2006a) reported the evaluation of enzymic potential for biotransformation of isoflavone phytoestrogen in soymilk by *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus casei*. Similary, Chien et al., (2006) studied the transformation of isoflavones during

the fermentation of soymilk with lactic acid bacteria and bifidobacteria. However, in these studies, the rate of transformation of IG to aglycones was low. For instance, only 6.4% of the total IG in soymilk was fermented by *B. longum* after 32 h of fermentation at 37 ^{o}C (Chien et al., 2006). In addition, the fermented soymilk does not have good commercial value, as the taste is not pleasant due to the strong beany flavour.

To enhance the level of the biotransformation of IG to aglycones, which was reported low in the fermented products by bifidobacteria and as well as to improve the quality of fermented soymilk, the product could be supplemented with SMP. As a good source of lactose, SMP also contains several nutritious components such as amino acids. These nutritional components were reported to enhance the growth and metabolism of bifidobacteria (Kontula et al., 1999). In addition, lactose was considered as a bifidogenic factor which stimulates the growth of bifidobacteria (Dubey & Mistry, 1996). Consequently, bifidobacteria are expected to grow better in soymilk supplemented with SMP than soymilk alone. Hence, the level of transformation of IG to aglycones is expected to be higher. Moreover, the fermented soymilk supplemented with SMP may improve taste compared to the fermented soymilk alone as SMP may reduce the beany flavour.

In this study, SPI was used to make soymilk. SPI is made from defatted soy meal containing about 90% protein. After hydrolysis by probiotic organisms, SPI contains 18 amino acids such as tryptophan, arginine, glutamic acid, isoleucine, leucine, tyrosine, cysteine and valine (Nutrition Data, 2007). All these amino acids were reported to be either stimulatory or essential for growth of bifidobacteria (Poch & Bezkorovainy, 1988). Therefore, SPI may have an effect on the growth of bifidobacteria. In addition, SPI contains approximately 150 mg of isoflavones per 100 g powder, lower content of isoflavones than in soy flour due to the mild alkali extraction used in the production of SPI (Wang & Murphy, 1996). However, to date, there is no report about the biotransformation of IG and lactose utilisation by *Bifidobacterium* in soy milk supplemented with SMP. Therefore, the objectives of this study were to examine the biotransformation of IG to aglycones by *Bifidobacterium* in SSM and to

assess the influence of SMP supplementation and lactose utilisation on the growth and acidification of *Bifidobacterium*.

5.2.2 Materials and Methods

5.2.2.1 Isoflavone compounds and other chemicals

Isoflavone compounds and other chemicals are described as section 4.1.2.1. Skim milk powder was from Murray Goulburn Co-Operative Company (Brunswick, Vic, Australia).

5.2.2.2 Bifidobacteria

Bifidobacterium strains A and B were used in this study. The strain numbers are not disclosed due to confidential reasons. Pure cultures of the 2 strains were obtained from the Victoria University Culture Collection (Werribee, Vic, Australia). Purity of cultures was checked, and both organisms were stored at -80 ^{o}C in 40% (v/v) sterile glycerol.

5.2.2.3 Fermentation of SSM, SM and RSM by probiotics

The 2 probiotic strains, *B. animalis* A and *B. animalis* B, were activated in De Mann Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK) (pH adjusted to 6.7 using 5M NaOH) successively twice at 37 ^{o}C for 20 h. Filter-sterilized L-cysteine.HCl solution was added to the medium at the final concentration 0.05% (w/v) to lower the oxidation-reduction potential and to enhance the growth of anaerobic bifidobacteria. The third transfer was carried out in SSM prepared from 4% (w/v) SPI supplemented with 12% (w/v) SMP, SM prepared from 4% SPI (w/v), and RSM prepared from 12% (w/v) SMP. Three milk streams were prepared intentionally with different total solid content since our objective was to keep the concentration of isoflavone compounds constant in the three media of medium SM, RSM, and SSM (they all contained 4% (w/v) of SPI), so that the transformation levels of isoflavone glucosides to aglycones in the three media by the same probiotic organism could compare together. One litre of each media was individually inoculated with 1% (v/v) of the active culture of probiotic and incubated at 37 ^{o}C for 24 h. Aliquots of 100 mL were withdrawn aseptically at 0, 6, 12, 18 and 24 h

of incubation for enumeration of viable probiotic populations, determination of pH and quantification of lactose then freeze-dried using a Dynavac freeze-dryer (model FD 300; Rowville, Vic, Australia) for quantification of isoflavone.

5.2.2.4 Enumeration of viable microorganisms

The spread plate method was used for enumeration of viable populations of *Bifidobacterium*. MRS agar supplemented with 0.05% (w/v) of L-cysteine.HCl was used for enumeration. One milliliter of serial dilutions at 6, 12, 18 and 24 h was aseptically spread on to the plates and incubated at 37 ^{o}C in an anaerobic jar (Becton Dickinson Microbiology System, Sparks, MD, USA) with a gas generating kit (Oxoid Ltd., Hampshire, UK). Colony counts between 25-250 were enumerated.

5.2.2.5 Determination of pH

Determination of pH is described as section 4.1.2.4

5.2.2.6 Determination of lactose contents

Determination of lactose content is described as section 5.1.2.4

5.2.2.7 Determination of isoflavone contents

Determination of isoflavone contents is described as section 3.2.5, 3.2.6 and 4.1.2.6

5.2.2.8 Statistical analysis of data

Statistical analysis of data is described as section 4.1.2.7

5.2.3 Results and Discussion

5.2.3.1 HPLC analysis of isoflavones

The HPLC chromatogram and the retention time of 14 standard isoflavones and the internal standard are shown in Figure 3.1. Normally, daidzin and glycitin are co-eluted as their chemical structures are similar. However, the co-elutions were resolved using

the gradient method reported in this study. The order of elution of the isoflavones depended on the polarity and hydrophobic interaction with the HPLC column (Tsangalis et al., 2002).

5.2.3.2 Lactose utilisation and pH of RSM and SSM fermented by B. animalis A & B

The pH of RSM and SSM and the lactose utilised during the fermentation by *B. animalis* A and B are shown in Figures 5.6 and 5.7. As shown in Figure 5.6, the pH of SM fermented by *B. animalis* A decreased slightly from 6.8 to 6.3 during 24 h of fermentation since the SPI contains very little carbohydrate (1%) (Soyfoods Association of North America, 2004). The results are in agreement with Tsangalis and Shah (2004) who reported the pH of soymilk made from 4% SPI to be at 6.3 at 24 h of fermentation by *B. longum* 1941.

As shown in Figure 5.6, the initial pH of RSM, SSM and SM was 6.89, 6.25 and 6.80, respectively, and these were within the optimum range for the growth of bifidobacteria (Shah, 2006). The initial lactose contents of the RSM and SSM were 55.28 and 52.85 mg/mL, respectively. The reduction in lactose content was due to fermentation by bifidobacteria. *Bifidobacterium animalis* A fermented lactose at a higher level in SSM than in RSM entire incubation but significantly higher ($P < 0.05$) at 24 h of incubation. 30.5% of lactose was utilised from RSM compared to 40.0% from SSM. As a result, pH of SSM was lower (3.80) than that in RSM (4.00) (Figure 5.6).

Similarly, as shown in Figure 5.7, *B. animalis* B also lowered the pH of SM from 6.8 to 6.05 during 24 h of fermentation as small amount of carbohydrate source was reported to be present in SPI (Nutrition Data, 2007). After 18 h of fermentation, *B. animalis* B utilised more lactose in SSM than RSM. At 24 h of incubation, 18.09 mg/mL of lactose was utilised in SSM compared to 16.02 mg/mL in RSM. Consequently, pH in SSM was lower (3.96) than that in RSM (4.50) (Figure 5.7).

As illustrated in Figures 5.6 and 5.7, both strains of *B. animalis* fermented more lactose in SSM than that in RSM, thus there was a greater decrease in pH in SSM than in RSM. This suggests that soymilk supplementation enhanced the lactose utilising ability of *B. animalis*. The presence of some essential amino acids in SPI such as tryptophan, isoleucine, leucine, cysteine, tyrosine, valine and glutamic acid may have enhanced the lactose metabolism (Nutrition Data, 2007).

5.2.3.3 Viable probiotic organisms

Figures 5.8 and 5.9 show the viable number (log CFU/mL) in SM, RSM and SSM during fermentation by *B. animalis* A and B. As shown in Figure 5.8, *B. animalis* A showed the poorest growth in SM although the viable counts increased steadily from 6.46 to 7.12 log CFU/mL during 24 h of incubation. Similarly, as shown in Figure 5.9, *B. animalis* B also showed the weakest growth in SM and after 24 h of incubation, the maximum viable counts only reached 6.94 log CFU/mL. *Bifidobacterium animalis* A exhibited the strongest growth in RSM (Figure 5.10). At 12 h of incubation the viable microbial number reached maximum at 9.74 log CFU/mL followed by a decline in the growth thereafter possibly due to a drop in pH in the media. *Bifidobacterium animalis* A exhibited a significantly higher ($P < 0.05$) growth in SSM than that in SM, but slightly lower than that in RSM during 24 h of incubation. At 12 h of incubation, the viable count of *B. animalis* A in SSM was 8.66 log CFU/mL compared to 9.74 log CFU/mL in RSM and 6.32 log CFU/mL in SM.

Bifidobacterium animalis B also exhibited an excellent growth in the RSM. The growth in SSM was slightly lower than that in RSM but considerably higher than in soymilk during 24 h of incubation. For instance, at 12 h, the viable microbial numbers in RSM, SSM and SM were 8.95, 8.64 and 6.52 log CFU/mL, respectively.

It appears that the presence of SPI reduced the viable population of *Bifidobacterium*. In contrast, adding SMP to soymilk significantly increased the viable counts of both *B. animalis* A and B as SMP contained lactose as well as other essential nutrients. *Bifidobacterium animalis* A showed higher viable population than those of *B. animalis* B in all three types of media in most time of incubation.

5.2.3.4 Biotransformation of IG in SM by B. animalis

The moisture content of SPI powder was 4.5 ± 0.1% and that of freeze-dried samples ranged from 1.95 - 2.02%. There were no significant ($P > 0.05$) differences in moisture contents of the freeze-dried samples. Therefore, it was assumed that there was no effect of the moisture content on the estimation of isoflavones. Tables 5.5, 5.6 and Figure 5.10 show the transformation of IG in SM by *B. animalis* A and B. There were only 8 isoflavone compounds detected in the SM (Table 5.5). Genistein was the only aglycone found in the medium at time 0, and it was present at very low concentration (4.50 ± 0.32 mg/ 100 g of freeze-dried sample), which was approximately 2.9% of total isoflavones. King & Bignell (2000) and Nakamura et al. (2001) reported that the aglycone contents were very minor in amount compared with IG. In the IG group, genistin and acetyl glycitin were not detected. The total initial IG were 148.81 ± 2.88 mg/ 100 g of freeze-dried sample with malonyl- and acetyl genistin as the dominant compounds. As Wang & Murphy (1996) reported, the mild alkali extraction in the production of SPI causes isoflavones losses of 53%. The absence of these isoflavone compounds is also possibly due to losses during the processing of SPI.

As shown in Table 5.5 and Figure 5.10 the concentration of IG decreased steadily and that of the aglycones increased during the fermentation. After 18 h of fermentation, aglycones produced were fairly stable. There was no significant ($P > 0.05$) difference between the aglycone contents produced at 18 and 24 h. At 6 h of incubation, glycitin and malonyl glycitin were hydrolysed completely. At 24 h, the 4 IG namely daidzin, malonyl daidzin, malonyl genistin and acetyl genistin were still detected while other IG were completely hydrolysed. The total aglycones concentration was 62.48 mg and 74.3% of the total IG was biotransformed to aglycones.

In general, *B. animalis* B hydrolysed IG to aglycones in SM with similar level to that by *B. animalis* A (Table 5.6), except for the first 6 h of incubation. For instance, at 18 h, 71.6% of IG were hydrolysed by *B. animalis* B compared with 70.6% by *B. animalis* A and at 24 h of incubation, 74.4% of IG were fermented by *B. animalis* A compared with that of 72.8% by *B. animalis* B (Figure 5.10). As a result, the total aglycones produced were 62.48 and 60.45 (mg/100g of freeze-dried sample) for *B. animalis* A and B,

respectively. Tsangalis et al. (2002) reported that 57.8% of IG in the plain soymilk were fermented by *B. pseudolongum*-a but only 9.8% of IG were fermented by *B. pseudolongum*-b at 24 h. Chien et al. (2006) also studied the biotransformation of IG to aglycones. In their study, 6.4% of IG in soymilk were fermented by *B. longum* at 32 h of incubation. In our study, both strains of *B. animalis* showed high level of biotransformation of IG compared to other bifidobacteria reported.

5.2.3.5 Biotransformation of IG in SSM by B. animalis

Tables 5.7 and 5.8 present the fermentation of IG in SSM by *B. animalis* A and B. Similar to the SM, there were 8 isoflavone compounds detected in SSM. Genistein was the only aglycone found in SSM at time 0, and it was present at very low concentration of 1.26 ± 0.08 mg/100 g of freeze-dried sample, which is approximately 3.6% of total isoflavones. The initial total IG was 34.11 ± 1.59 mg/ 100 g of freeze-dried sample. As shown in Table 5.7, daidzin, glycitin and acetyl daidzin were fermented completely by *B. animalis* A at 6 h of incubation. There was no significant ($P > 0.05$) increase in the production of aglycones after 18 h of incubation. At 24 h of incubation, 84.0% of IG were bio-transformed to aglycones including daidzein, glycitein and genistein at the concentration of 4.95, 0.82 and 10.39 mg/100 g of freeze-dried sample, respectively.

Bifidobacterium animalis B hydrolysed higher level of IG to aglycones than *B. animalis* A in SSM (Table 5.7). At 6 h of incubation, *B. animalis* B fermented 72.4% of IG, considerably higher than that (58.8%) by *B. animalis* A. However, after 18 h of incubation, similar to *B. animalis* A, the hydrolysis of IG became fairly stable and the total amount of aglycones produced were not significantly ($P > 0.05$) different between 18 and 24 h of incubation. At 24 h of incubation, the total aglycones produced were 15.94 mg/100g of sample by de-conjugating 85.4% of total IG.

Therefore, *B. animalis* A and B showed the similar trend and level in the biotransformation of IG to aglycones after 12 h of incubation in both SSM and SM. However, in the first 12 h of incubation *B. animalis* B appeared to ferment IG faster and noticeably higher than *B. animalis* A.

It was obvious that the level of biotransformation of IG to aglycones in SSM was significantly higher than that in SM. Hence, our results suggested that SMP played a

key role in enhancing the biotransformation of IG to aglycones by *Bifidobacterium*. According to Tsangalis et al. (2002), Otieno et al. (2005) and Chien et al., (2006), β-glucosidase produced by *B. animalis*, played a key role in breaking down the β-glucosidic bond in IG to liberate the biologically active aglycones. On the other hand, *Bifidobacterium* also produces a considerable amount of β-galactosidase, which could be able to hydrolyse IG to aglycones (Shah & Jelen, 1990). SMP contains approximately 50% of lactose, which is considered as bifidogenic (Shah, 1993; Poch & Bezkorovainy, 1988). This may have enhanced the production of β-galactosidase and β-glucosidase activity hence greater biotransformation of IG to aglycones.

5.2.4 Conclusions

Our results suggested that the supplementation with SMP enhanced the biotransformation level of IG to IA by both bifidobacteria and lactobacilli. However, enhancing effect on lactobacilli (from 13.9-19.0%) was higher than that on bifidobacteria (10-12.6%). That is possibly due to the different path ways of lactose utilisation of lactobacilli and bifidobacteria in which the activities of β-galactosidase and β-glucosidase were not similar.

It was noticed that the presence of SMP in the medium SSM improved the growth of all the four strains of probiotic organisms from 0.8-1.0 log CFU/mL while the viable count of bifidobacteria increased in a range of 2.0 -3.0 log CFU/mL. That would improve the health benefits of the fermented product values as well.

In addition, the presence of SPI stimulated the lactose utilisation in SMP, and the effect slightly varied with the probiotic organism strains in a range 3 – 5 mg/mL.

Table 5.5 Biotransformation of IG to aglycones in SM by *B. animalis* A and B

Isoflavone (mg/100 g of freeze-dried sample)	*B. animalis* A					*B. animalis* B				
	0 h	*6 h*	*12 h*	*18 h*	*24 h*	*0 h*	*6 h*	*12 h*	*18 h*	*24 h*
Daidzin	14.03 ± 0.70^{a}	9.34 ± 0.75^{b}	6.01 ± 0.54^{c}	3.21 ± 0.25^{d}	2.58 ± 0.21^{d}	14.03 ± 0.70^{a}	6.59 ± 0.42^{b}	3.20 ± 0.24^{c}	ND	ND
Glycitin	6.13 ± 0.10	ND	ND	ND	ND	6.13 ± 0.10	ND	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	24.49 ± 1.69^{a}	17.04 ± 1.04^{b}	8.31 ± 0.71^{c}	5.50 ± 0.42^{cd}	5.50 ± 0.35^{d}	24.49 ± 1.69^{a}	5.73 ± 0.37^{b}	4.00 ± 0.32^{b}	ND	ND
Malonyl glycitin	3.02 ± 0.07	ND	ND	ND	ND	3.02 ± 0.07	ND	ND	ND	ND
Acetyl daidzin	6.41 ± 0.19^{a}	6.20 ± 0.51^{b}	ND	ND	ND	6.41 ± 0.19^{a}	ND	ND	ND	ND
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl genistin	67.23 ± 2.02^{a}	29.35 ± 1.37^{b}	22.61 ± 1.57^{c}	20.25 ± 2.12^{c}	15.33 ± 1.04^{d}	67.23 ± 2.02^{a}	31.49 ± 1.75^{b}	29.87 ± 1.54^{bc}	28.85 ± 1.65^{bc}	26.90 ± 1.82^{c}
Acetyl genistin	27.50 ± 1.63^{a}	14.60 ± 1.08^{b}	13.87 ± 1.21^{b}	14.85 ± 1.17^{b}	14.75 ± 1.11^{b}	27.50 ± 1.63^{a}	16.50 ± 1.06^{b}	13.45 ± 1.19^{b}	13.39 ± 1.08^{b}	13.54 ± 0.99^{b}
Total of IG	***148.81 ± 2.88^{a}***	***76.53 ± 4.75^{b}***	***50.80 ± 0.19^{c}***	***43.81 ± 0.28^{d}***	***38.16 ± 2.01^{e}***	***148.81 ± 2.88^{a}***	***60.31 ± 2.02^{b}***	***50.52 ± 0.43^{c}***	***42.24 ± 0.57^{d}***	***40.44 ± 2.81^{d}***
Daidzein	ND	6.32 ± 0.45^{a}	16.87 ± 1.25^{b}	19.75 ± 1.32^{b}	19.42 ± 1.24^{b}	ND	18.89 ± 1.25^{a}	20.89 ± 1.54^{a}	20.67 ± 1.29^{a}	21.40 ± 1.47^{a}
Glycitein	ND	3.65 ± 0.22^{a}	3.75 ± 0.25^{a}	3.75± 0.31^{a}	3.85 ± 0.54^{a}	ND	3.86 ± 0.28^{a}	3.75 ± 0.21^{a}	3.92 ± 0.27^{a}	3.78 ± 0.33^{a}
Genistein	4.50 ± 0.32^{a}	30.31± 2.46^{b}	31.97 ± 2.11^{b}	33.03 ± 2.58^{b}	39.21± 2.59^{c}	4.50 ± 0.32^{a}	29.80 ± 1.38^{b}	33.83 ± 2.14^{bc}	33.52 ± 1.78^{bc}	35.27 ± 2.50^{c}
Total of aglycones	***4.50 ± 0.32^{a}***	***40.28 ± 3.13^{b}***	***52.59 ± 3.61^{c}***	***54.53 ± 1.57^{cd}***	***62.48 ± 3.29^{d}***	***4.50 ± 0.32^{a}***	***52.55 ± 2.91^{b}***	***58.47 ± 0.39^{bc}***	***58.11 ± 2.80^{bc}***	***60.45 ± 3.64^{c}***

Results are expressed as mean ± standard error (n=3). One-way ANOVA was used to analyze the differences between means. Mean values in the same row for a particular *Bifidobacterium* with the same lowercase superscripts are not significantly different (P>0.05). ND: Not detected in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 μL.

Table 5.6 Biotransformation of IG to aglycones in SSM by *B. animalis* A and B

Isoflavone (mg/100 g of freeze-dried sample)	*B. animalis* A					*B. animalis* B				
	0 h	*6 h*	*12 h*	*18 h*	*24 h*	*0 h*	*6 h*	*12 h*	*18 h*	*24 h*
Daidzin	3.37 ± 0.15^{a}	ND	ND	ND	ND	3.37 ± 0.15^{a}	ND	ND	ND	ND
Glycitin	1.19 ± 0.05	ND	ND	ND	ND	1.19 ± 0.05	ND	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl daidzin	4.81 ± 0.25^{a}	0.88 ± 0.07^{b}	0.47 ± 0.03^{c}	ND	ND	4.81 ± 0.25^{a}	ND	ND	ND	ND
Malonyl glycitin	1.06 ± 0.05^{a}	1.00 ± 0.12^{a}	1.00 ± 0.08^{a}	ND	ND	1.06 ± 0.05^{a}	ND	ND	ND	ND
Acetyl daidzin	1.52 ± 0.08^{a}	ND	ND	ND	ND	1.52 ± 0.08^{a}	ND	ND	ND	ND
Acetyl glycitin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Malonyl genistin	16.13 ± 0.72^{a}	8.49 ± 0.56^{b}	6.82 ± 0.65^{bc}	6.28 ± 0.51^{c}	5.47 ± 0.47^{c}	16.13 ± 0.72^{a}	6.09 ± 0.52^{b}	6.07 ± 0.41^{b}	5.95 ± 0.45^{b}	4.97 ± 0.35^{b}
Acetyl genistin	6.03 ± 0.29^{a}	3.69 ± 0.27^{b}	2.33 ± 0.21^{c}	ND	ND	6.03 ± 0.29^{a}	3.32 ± 0.21^{b}	1.95 ± 0.17^{c}	ND	ND
Total of IG	***34.11 ± 1.59^{a}***	***14.06 ± 0.78^{b}***	***10.62 ± 0.97^{c}***	***6.28 ± 0.51^{d}***	***5.47 ± 0.47^{d}***	***34.11 ± 1.59^{a}***	***9.41 ± 0.31^{b}***	***8.02 ± 0.58^{bc}***	***5.95 ± 0.45^{c}***	***4.97 ± 0.35^{c}***
Daidzein	ND	3.37 ± 0.19^{a}	3.46 ± 0.24^{a}	4.60 ± 0.35^{a}	4.95 ± 0.35^{b}	ND	4.18 ± 0.21^{a}	4.28 ± 0.24^{a}	4.47 ± 0.33^{ab}	5.19 ± 0.37^{b}
Glycitein	ND	0.38 ± 0.05^{a}	0.53 ± 0.09^{a}	0.55 ± 0.07^{a}	0.82 ± 0.11^{b}	ND	0.50 ± 0.08^{a}	0.58 ± 0.04^{a}	0.45 ± 0.08^{a}	0.91 ± 0.07^{b}
Genistein	1.26 ± 0.08^{a}	6.79 ± 0.52^{b}	8.38 ± 0.72^{bc}	9.67 ± 0.56^{cd}	10.39 ± 1.02^{d}	1.26 ± 0.08^{a}	9.07 ± 0.87^{b}	9.85 ± 0.65^{b}	10.95 ± 0.99^{b}	11.10 ± 1.02^{b}
Total of aglycones	***1.26 ± 0.08^{a}***	***10.54 ± 0.38^{b}***	***12.38 ± 1.05^{bc}***	***14.82 ± 0.98^{cd}***	***16.16 ± 0.78^{d}***	***1.26 ± 0.08^{a}***	***13.74 ± 0.58^{b}***	***14.71 ± 0.37^{b}***	***15.87 ± 0.74^{bc}***	***17.20 ± 1.46^{c}***

Results are expressed as mean ± standard error (n=3). One-way ANOVA was used to analyze the differences between means. Mean values in the same row for a particular *Bifidobacterium* with the same lowercase superscripts are not significantly different (P>0.05). ND: Not detected in 1 g freeze dried soymilk used to extract isoflavones with a sample injection volume of 20 µL.

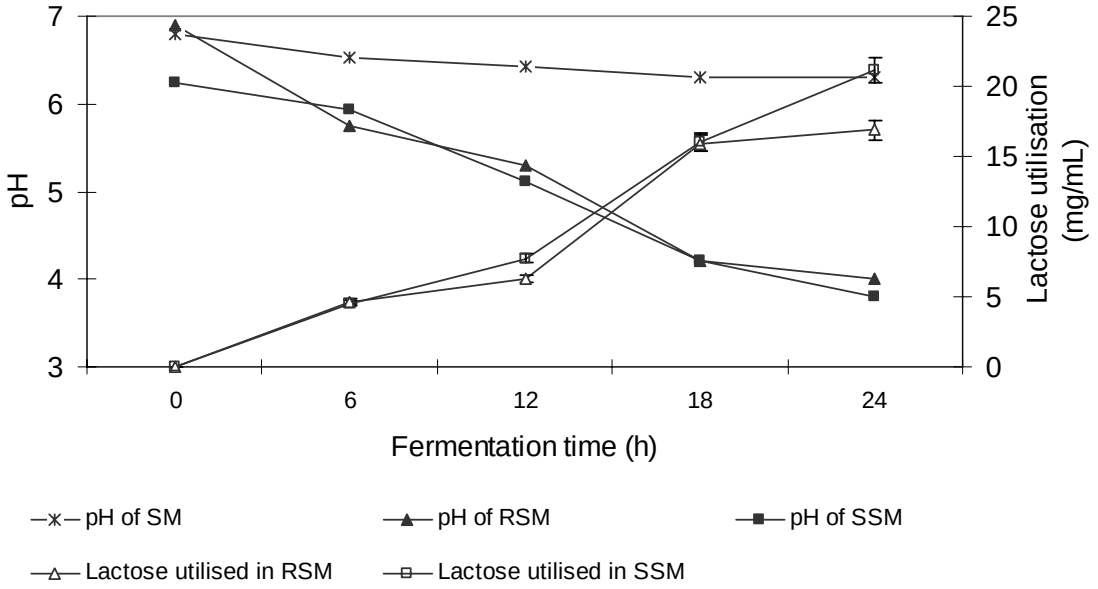


Figure 5.6 pH values and lactose utilisation (mg/mL) of RSM, SM and SSM fermented by *B. animalis* A

Results are expressed as mean ± standard error (n = 3)

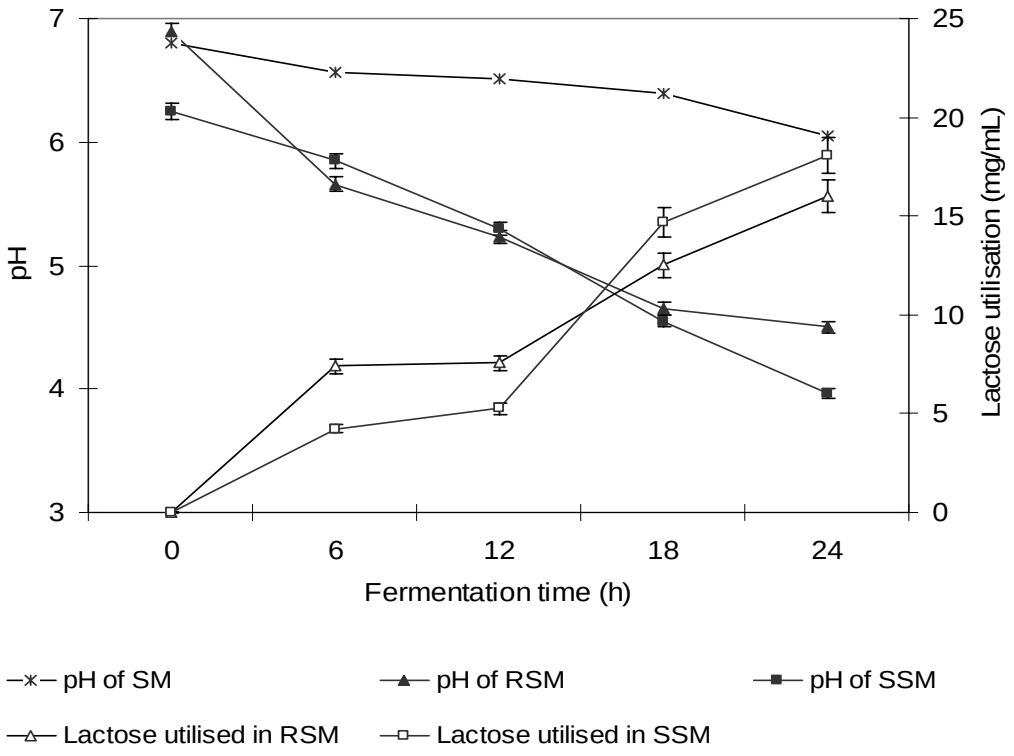


Figure 5.7 pH values and lactose utilisation (mg/mL) of RSM, SM and SSM fermented by *B. animalis* B

Results are expressed as mean ± standard error (n = 3)

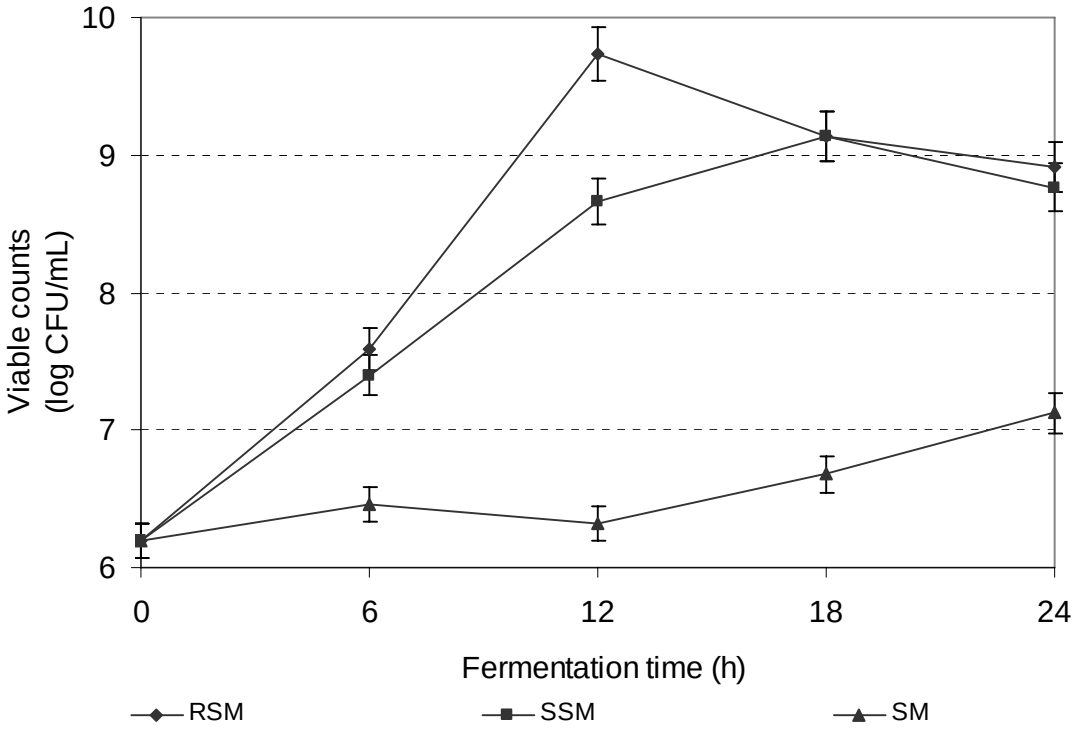


Figure 5.8 Viable microbial counts (log CFU/mL) of *B. animalis* A in RSM, SM and SSM fermented for 24 h at 37 ^{o}C.

Results are expressed as mean ± standard error (n = 3)

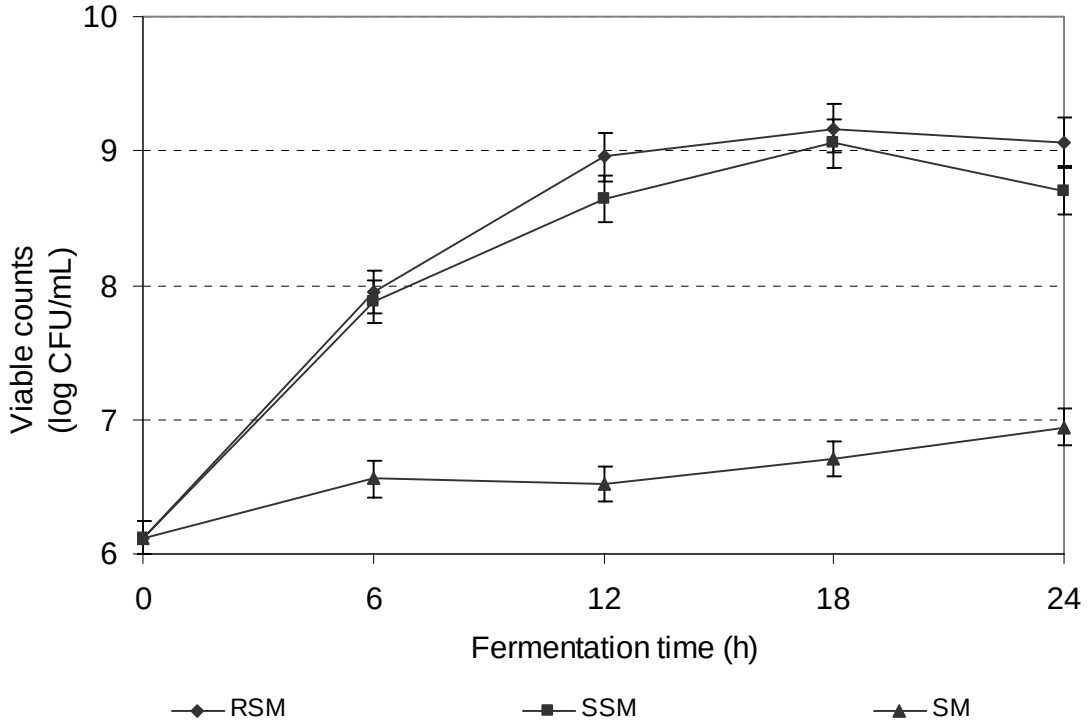


Figure 5.9 Viable microbial counts (log CFU/mL) of *B. animalis* B in RSM, SM and SSM fermented for 24 h at 37 ^{o}C.

Results are expressed as mean ± standard error (n = 3)

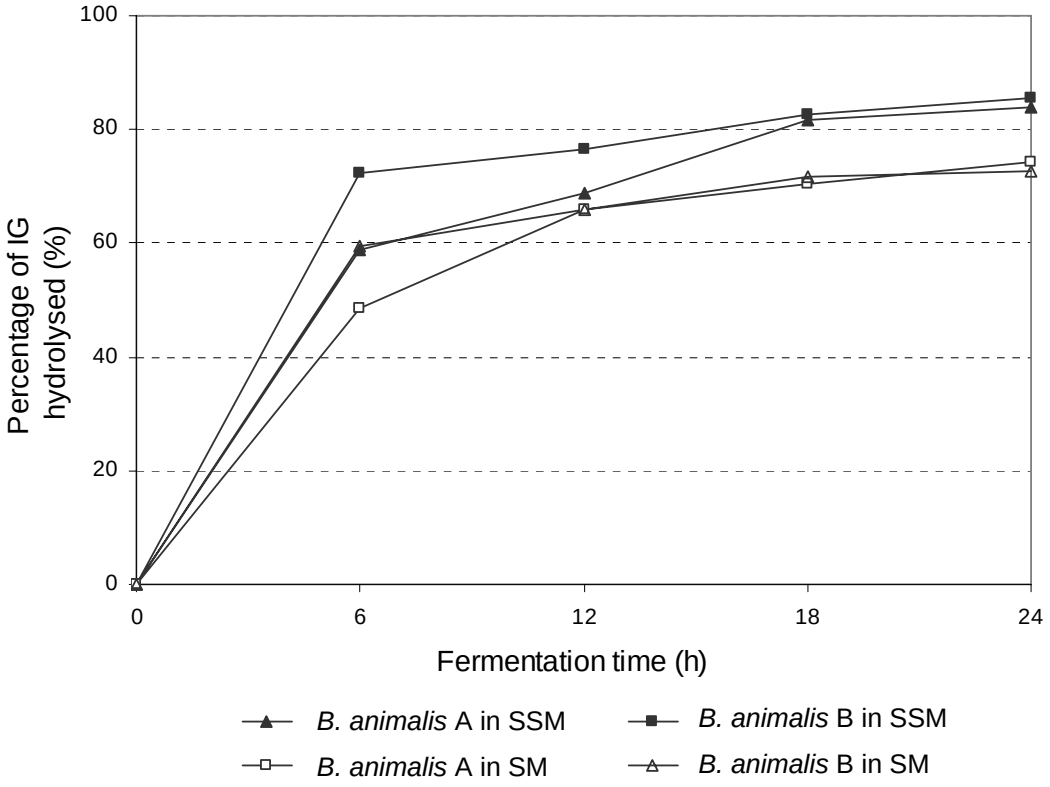


Figure 5.10 Biotransformation (%) of IG to aglycones in SSM and SM by *B. animalis* A and B.

Results are expressed as mean ± standard error (n = 3)

Chapter 6.0

Performance of starter cultures in yogurt supplemented with soy protein isolate and biotransformation of isoflavones during storage period

This chapter has been published:

Pham, T. T., & Shah, N. P. (2009). Performance of starter in yogurt supplemented with soy protein isolate and biotransformation of isoflavones during storage period. **Journal of Food Science**, 74, M190-M195

6.1 Introduction

Traditionally, yogurt is perceived as a nutritious food product while soy yogurt (or soygurt) is a relatively new product and was just introduced several decades ago by Ariyama (1963). Soygurt would contain a considerable amount of isoflavones which are phytochemical compounds that have caught the attention of many researchers recently. As described in the previous chapters 4.0 and 5.0, isoflavone compounds have been reported to provide many health benefits. Therefore, to take advantage of isoflavone related health promoting properties, supplementation with soymilk to yogurt mix could be a good approach. Soy protein isolate (SPI) is utilised widely due to their high score of protein digestibility corrected amino acid. Since cow milk is generally considered to be lacking in several essential amino acids such as isoleucine, the supplementation with this amino acid through SPI could enhance the nutritive value of the product (Gomes et al., 1998; Hofman & Thonart, 2001; Nutrition Data, 2007). In addition, the supplementation is also expected to improve the growth of the yogurt starter micoorganism by providing them with an appropriate growth medium. Furthermore, SPI is able to play a role as an emulsifier in yogurt as it can form a stable emulsion and foam in fermented dairy products, hence it can possibly enhance the texture of yogurt (Snyder & Kwon, 1987). Most importantly, as shown in the chapter 3.0, SPI contains a moderate amount of isoflavone compounds (12-102 mg/100 g). However, the isoflavone compounds predominantly exist in SPI as well as in other non-fermented soy products in inactive forms of isoflavone glycosides (IG). Because of conjugation with a β-glycoside molecule, they are not able to be absorbed through the human gut wall therefore IG do not possess any estrogenic effects nor do they provide other health benefits such as anti-breast and prostate cancer effect (Hughes et al., 2003). Only isoflavone aglycones (IA), which are the forms of IG freed from the β-glycoside molecule, provide health benefits. There are only three IA compounds including daidzein, glycitein and genistein which are found in SPI in minor concentration of 5 mg/100 g (Pham & Shah, 2007). However, in order to have beneficial effects, a considerable amount (30 - 40 mg/day) of IA in a food product is required (Malnig & Brown, 2007). Therefore, it is better to convert IG to IA prior in food products since IG

are hydrolysed to IA in the gastro-intestinal tract at a slow rate and depending on individuals (depending on diet, age, sex, location etc...)(Hughes et al., 2003; Sugano, 2005).

As described in Chapter 3.0, both β-glucosidase and β-galactosidase are also able to hydrolyse the β-glucosidic linkage in IG. The traditional yogurt starter including *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are able to produce high level β-galactosidase (Vedamuthu, 2006). Hence, these organisms have the potential for transformation of IG to IA during the incubation, resulting in the fermented product enriched with IA. In addition, in low pH condition of yogurt, some IG such as malonyl daidzin are partly hydrolysed to daidzein (Mathias et al., 2006).

To date, there is very little information about the influence of the supplementation with SPI on the biotransformation of IG to IA in yogurt as well as during a cold storage period. Therefore, the objectives of this study were to investigate the influence of the supplementation with SPI on the performance of yogurt starter including *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* on (i) lactose utilisation, (ii) organic acids production (iii) survival of the starter organisms and (iv) the biotransformation of IG to IA in SY by the yogurt starter during the storage period of 28 days at 4 ^{o}C.

6.2 Materials and Methods

6.2.1 Chemicals

Isoflavone compounds and other chemicals are described as section 4.1.2.1. Reinforced clostridial agar and M17 agar were from Amyl Media (Danenong, Vic, Australia). Skim milk powder (SMP) was from Murray Goulburn Co-operative Ltd. (Brunswick, Vic, Australia).

6.2.2 Starters and fermentation

Pure frozen culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (Lb 11842) was obtained from Australian Starter Culture Research Centre (Werribee, Vic,

Australia). Pure strain of *Streptococcus thermophilus* ST 1342 *(S. thermophilus* 1342) in frozen form was from the Victoria University Culture Collection (Werribee, Vic, Australia). The two organisms were separately activated in de Mann Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK) by growing successively twice at 37 ^{o}C for 20 h. Two litres of reconstituted SMP (12%, w/w) with or without the supplementation with 4% (w/w) SPI were prepared for making supplemented yogurt (SY) and the control yogurt (USY), respectively. The two milk streams were prepared intentionally in different solid contents in order to have the same initial lactose content in them. (Both of the streams contained 12% of SMP). Our objective was to investigate the performance of the starter on SY, where isoflavone was supplemented.

The mixes were heat treated in a water bath (model NB 6T-10935; Thermoline Scientific, Australia) at 85 ^{o}C for 30 min, followed by cooling to 42 ^{0}C and each mix was aseptically inoculated with 1% each of Lb 11842 and *S. thermophilus* 1342. Inoculated mixes were then poured into 50 mL sterile cups with lids and incubated at 42 ^{0}C until the pH of the products reached 4.50 ± 0.10. The finished yogurts were immediately cooled in an ice bath and then stored at 4 ^{o}C for 28 d. All the experiments were carried out in duplicate.

6.2.3 Determination of pH

The pH of the SY and USY was monitored at the end of the fermentation (0 d), and at 7 d interval during 28 d of the storage using a microprocessor pH meter (model 8417; Hanna Instruments, Singapore) at 20 °C after calibrating with fresh pH 4.0 and 7.0 standard buffers.

6.2.4 Enumeration of viable micro-organisms

One gram sample of SY and USY was taken at 0, 7, 14, 21 and 28 d of storage and serial dilutions were prepared in 0.15% (w/v) peptone water. The colonies of Lb 11842 and *S. thermophilus* 1342 were enumerated using the pour plate technique as described previously (Dave & Shah, 1996). Briefly, M17 agar was used for the selective enumeration of *S. thermophilus* 1342 and the plates were incubated aerobically at 37 ^{o}C

for 72 h. Reinforced clostridial agar (pH 5.3) was used for the enumeration of Lb 11842 and the microorganism was incubated anaerobically at 37 ^{o}C for 72 h. Plates showing colonies between 25 to 250 were enumerated and recorded as colony forming unit (CFU) per gram of yogurt.

6.2.5 Determination of organic acids

Lactic and acetic acids were determined using the method described by Donkor et al. (2005) with some modifications. Briefly, 0.5 gram of the yogurt sample was mixed with 25 µL of 15.5 M nitric acid and then diluted with 0.8 mL of 5 mM H_2S0_4. The mixture was centrifuged at 14,000 x g for 30 min using an Eppendorf 5415C centrifuge (Crown Scientific, Melbourne, Australia) to remove proteins. The supernatant was filtered through a 0.45 µm membrane filter (Phenomenex, Lane Cove, NSW, Australia) into a HPLC vial. The HPLC systems were Varian HPLC (Varian Analytical Instruments, Walnut Creek, CA, USA) and an Aminex HPX-87H, 300 x 7.8 mm ion-exchange column (Biorad Life Science Group, Hercules, CA, USA). The column was maintained at 65 ^{o}C by a column heater (serial No. 2451; Timberline Instrument Inc., Boulder, CO, USA). Sulphuric acid (5 mM) was used as a mobile phase at a flow rate of 0.6 mL/min. The level of organic acids was quantified based on standard curves prepared using standard solutions.

6.2.6 Determination of lactose content

Determination of lactose was described as section 5.1.2.4

6.2.7 Determination of isoflavone contents

Fifty of SY was taken at the end of the fermentation (0 d) and at 7, 14, 21 and 28 d of the storage and freeze-dried using a Dynavac freeze-dryer (model FD 300; Rowville, Vic, Australia) for quantification of isoflavones. The next steps were described as sections 3.2.5, 3.2.6 and 4.1.2.6.

6.2.8 Statistical analysis of data

The fermentation trials were carried out in duplicates and all analyses were performed in triplicate. The data were analysed using one-way analysis of variance (ANOVA) at 95% confidence intervals using Microsoft Excel Statpro as described by Allbright et al. (1999). ANOVA data with a $P < 0.05$ was classified as statistically significant.

6.3 Results and Discussion

6.3.1 The influence of the supplementation with SPI on the performance of yogurt starter during storage at 4 ^{o}C

6.3.1.1 Lactose metabolism

Figure 6.1 presents the lactose concentration in both SY and USY during the storage period. As shown in the figure, the initial lactose contents in SY and USY were 43.95 and 47.03 mg/g, respectively, although the lactose contents in the mix prepared for SY and USY were at 65.42 mg/g (data not shown). Hence, during the fermentation of the yogurt mixes, significantly ($P<0.05$) higher amount of lactose was utilised by *S. thermophilus* 1342 and Lb 11842 in SY than that in USY by 4.7%. This result suggests that the supplementation with SPI to SY significantly promoted the lactose metabolism by the yogurt starter during the fermentation process. This could be due to the enrichment of nitrogen source through SPI for the yogurt starter. Since SPI contains 18 amino acids including 11.0 mg/g of tryptophan and 65.9 mg/g of arginine which are complementary to the inadequate nitrogen source such as tryptophan and arginine in SMP (Nutrition Data, 2007; Poch & Bezkorovainy, 1988)). To synthesise enzymes involved in lactose utilisation, several amino acids are needed. Consequently, the rich source of amino acids released from SPI during the hydrolysis by probiotic organisms in SY could help the yogurt starter to utilise lactose more efficiently (Vedamuthu, 2006).

It also appears that, during the entire storage period, the lactose content in SY was always significantly ($P < 0.05$) lower than that in USY. For instance, at 21 d of the storage period, the lactose content in SY was 41.72 mg/g yogurt compared to 45.01 mg/

g in USY. However, the amounts of lactose utilised by the yogurt starter during the entire 28 d of the storage period in SY and USY were similar (2.21 and 2.08 mg/g yogurt, respectively). The reason is possibly due to the inactive status of enzymes such as lactase that are involved in lactose utilisation in low pH and temperature condition of storage period in both SY and USY.

6.3.1.2 Organic acids production

Figure 6.2 illustrates the organic acids concentration including lactic and acetic acids, and the pH values of SY and USY during the storage period. Although other organic acids such as orotic, citric, pyruvic, uric and formic acids are present in yogurt, however, they are found in very low concentration (Fernandez-Garcia & McGregor, 1994). Lactic and acetic acids are the two dominant organic acids in yogurt. Especially, lactic acid is used as an indicator to evaluate the fermentation of the yogurt starter (Vedamuthu, 2006). As shown in Figure 6.2, the acetic acid concentration in SY was insignificantly ($P>0.05$) higher than that in USY. In contrast, the lactic acid concentration in SY is insignificantly ($P>0.05$) lower compared to that in USY. As a result, the ratio of lactic acid to acetic acid in SY was lowering than that in USY. At 28 d of storage period, the ratios were 8.61 and 10.33, respectively. Hence, our study suggests that the presence of SPI slightly altered the production of lactose metabolism of the yogurt starter. This was in agreement with the study of Gomes et al. (1998), who also reported that the production of lactic acid decreased and acetic acid increased in milk supplemented with a rich protein source, milk hydrolysates, for fermentation by lactic acid bacteria.

As shown in Figure 6.2., the pH values in SY were always lower than those in USY. Our study suggests the supplementation with SPI could reduce the fermentation time of yogurt. In fact, after the same fermentation time of 8 h, the pH values of SY and USY were 4.55 and 4.60, respectively. The reason might be that the reconstituted SMP exhibited a stronger buffering capacity than reconstituted SPI. The maximum buffering capacity of milk is around 5.1, considerably close to the pH zone of yogurt at 4.6 (Figure 6.2) (Chandan, 2006). Consequently, the pH of USY was in range of 4.35 to 4.60 compared to the range of 4.15 to 4.55 in SY, during 28 d of the storage period.

6.3.1.3 Viability of yogurt starter

Figure 6.3 presents the viability of the yogurt starter including Lb 11842 and *S. thermophilus* 1342 in SY and USY during the storage period at 4 ^{o}C. For the first 7 d of the storage period, the survival of the yogurt starter in SY was significantly higher ($P<0.05$) than that in USY. The reason could be the yogurt starter was provided more nutritious by SY than USY. However, from 14 d of the storage period, the viability of both *S. thermophilus* 1342 and Lb 11842 in SY were significantly lower ($P < 0.05$) than those in the control USY. In addition, during the storage period, the viability of *S. thermophilus* 1342 and Lb 11842 in SY decreased by 0.94 and 0.61 log CFU/g, respectively, compared to 0.36 and 0.27 log CFU/g in USY. Thus, the pH values may play a key role in lowering the survival of the yogurt starter in SY since from 14 d of the storage period, pH of SY was 0.20 – 0.27 lower than that in USY (Figure 6.2). However, the viable counts of both *S. thermophilus* 1342 and Lb 11842 in SY were still in the range of 8.84-9.78 and 8.11-8.72 log CFU/g, respectively. Those were higher than the minimum concentration required at 7.0 log CFU/g to have health benefits (Frye, 2006). Although *S. thermophilus* 1342 and Lb 11842 are not classified as probiotic organisms, these bacteria can improve lactose digestion and may help promote a healthy immune system. Hence, it is desirable that they remain alive at a high concentration during storage in order to have beneficial effects (Zonis, 2007).

6.3.2 The biotransformation of IG to IA by the yogurt starters in SY during the storage period of 28 days at 4 ^{o}C

Table 6.1 presents the biotransformation of IG to IA by the yogurt starter in SY during the storage period of 28 days. Also, the HPLC chromatograms and the retention times of 12 standard isoflavone compounds and those in SY at 28 d of the storage period at 4 ^{o}C are shown in Figure 3.1 and Figure 6.4, respectively. As seen in Table 6.1, at 0 d of storage period (i.e. right after the fermentation), the yogurt starters biotransformed 72.8% of the total IG in SY to their counterpart IA including daidzein, glycitein and genistein. Three IG including daidzin, glycitin and acetyl daidzin were transformed completely to IA during the incubation process of making yogurt. The total of other 4

IG (malonyl daidzin, malonyl glycitin, malonyl genistin and acetyl genistin) remained at low concentration at 9.32 mg/100 g of freeze-dried samples. Among 7 IG identified in the mix before fermentation, malonyl genistin and malonyl glycitin appeared to be converted to their IA counterparts at the lowest level. At the 28 d of the storage, only 59.5% of malonyl genistin and 42.6% of malonyl glycitin were converted to genistein and glycitein, respectively. After the fermentation, the total IA in SY increased from 1.35 to 15.02 mg/100 g of freeze-dried samples (Table 6.1). According to Malnig & Brown (2007), the consumption of 30-40 mg of IA per day would provide health benefits. In our study, the product SY contained a considerable amount of IA and a high concentration of live yogurt starter (Table 6.1 and Figure 6.2). In addition, *S. thermophilus* 1342 and Lb 11842 demonstrated high biotransformation ability compared to other lactic acid bacteria. For instance, *Lactobacillus acidophilus* and *Lactobacillus casei* converted only 60.1% and 47.5%, respectively, of IG to IA after 6 h of fermentation of reconstituted SMP supplemented with soymilk (Pham & Shah, 2008b). During the storage period, the biotransformation level of IG to IA increased slightly although the survival rate of both *S. thermophilus* 1342 and Lb 11842 remained high. There were no significant differences ($P > 0.05$) in the total residual IG and the total of IA produced during the entire 28 d of storage (Table 6.1). The biotransformation slightly increased from 72.8 to 75.5% during storage period. The reason is possibly due to the inactive status of two enzymes including β-glucosidase and β-galactosidase produced by *S. thermophilus* 1342 and Lb 11842 in a low pH condition (pH 4.15– 4.60) of SY since they are denatured at low pH (Figure 6.2). In addition, our result shows that the total IA only increased by 0.49 mg/100 g of freeze-dried sample during the entire storage period. This suggests that 4 IG and 3 IA found in SY were considerably resistant to the acidic condition in pH range of 4.15 – 4.55.

6.4 Conclusion

The supplementation with SPI to yogurt mix had a significant impact on the performance of the yogurt starter including *S. thermophilus* 1342 and Lb 11842. Although the supplementation with SPI altered the ratio of lactic acid acetic acid by decreasing the lactic acid content and increasing the concentration of acetic acid in SY,

it promoted the metabolism of lactose by the yogurt starter during the storage, especially from 14 d. Additionally, the yogurt starter appeared to have a good capability for biotransformation of IG to IA. Within only 8 h of incubation, 72.8% of the total IG was converted to IA, increasing the amount of IA by 11.1 times (by 13.67 mg/ 100 g of freeze-dried sample). Therefore, SY contained a high concentration of the live yogurt starter (8.11-8.84 log CFU/g), and a considerable amount of IA (15.02- 15.51 mg/100 g of freeze-dried sample) and may provide enormous health benefits.

Table 6.1 The biotransformation of IG to IA in SY by the yogurt starter during the storage at 4 ^{o}C for 28 d

Isoflavones mg/100 g freeze-dried sample)	Before fermentation	Storage time (d)				
		0	7	14	21	28
Daidzin	3.32 ± 0.18	ND	ND	ND	ND	ND
Glycitin	1.12 ± 0.07	ND	ND	ND	ND	ND
Genistin	ND	ND	ND	ND	ND	ND
Malonyl daidzin	4.91 ± 0.25 ^{a}	0.51 ±0.08 ^{b}	0.51 ± 0.10 ^{b}	0.41 ± 0.09 ^{b}	0.39 ± 0.09 ^{b}	0.41 ± 0.10 ^{b}
Malonyl glycitin	1.08 ± 0.05 ^{a}	0.66 ± 0.11 ^{b}	0.68 ± 0.12 ^{b}	0.65 ± 0.13 ^{b}	0.61 ± 0.08 ^{b}	0.62 ± 0.09 ^{b}
Malonyl genistin	16.13 ± 0.08 ^{a}	7.10 ± 0.40 ^{b}	6.94 ± 0.35 ^{b}	6.59 ± 0.28 ^{b}	6.56 ± 0.32 ^{b}	6.56 ± 0.31 ^{b}
Acetyl daidzin	1.52 ± 0.09	ND	ND	ND	ND	ND
Acetyl glycitin	ND	ND	ND	ND	ND	ND
Acetyl genistin	6.21 ± 0.25 ^{a}	1.05 ± 0.08	1.02 ± 0.10	1.01 ± 0.07	0.90 ± 0.08	0.85 ± 0.12
Total IG	**34.29 ± 0.47 ^{a}**	**9.32 ± 0.51 ^{b}**	**9.15 ± 0.67 ^{b}**	**8.66 ± 0.57 ^{b}**	**8.46 ± 0.23 ^{b}**	**8.44 ± 0.43 ^{b}**
Daidzein	ND	5.51 ± 0.30 ^{a}	5.46 ± 0.34 ^{a}	5.50 ± 0.27 ^{a}	5.55 ± 0.25 ^{a}	5.55 ± 0.20 ^{a}
Glycitein	ND	0.83 ± 0.11 ^{a}	0.81 ± 0.09 ^{a}	0.83 ± 0.11 ^{a}	0.85 ± 0.12 ^{a}	0.84 ± 0.09 ^{a}
Genistein	1.35 ± 0.10 ^{a}	8.68 ± 0.50 ^{b}	8.74 ± 0.54 ^{b}	9.02 ± 0.47 ^{b}	9.07 ± 0.35 ^{b}	9.12 ± 0.32 ^{b}
Total IA	**1.35 ± 0.10 ^{a}**	**15.02 ± 0.31 ^{b}**	**15.01 ± 0.97 ^{b}**	**15.35 ± 0.85 ^{b}**	**15.47 ± 0.72 ^{b}**	**15.51 ± 0.61 ^{b}**
Biotransformation level (%)	**0**	**72.8**	**73.3**	**74.7**	**75.3**	**75.5**

Results are expressed as mean ± standard error (n = 6). Data were analysed by means of one-way ANOVA. Mean values in the same row with the same lowercase superscripts are not significantly different (P > 0.05). IG: Isoflavone glycosides. IA: Isoflavone aglycones. ND: Not detected (the isoflavone content which was in 1 g freeze-dried sample used to extract isoflavones with an injection volume of 20 µL was lower than the detection limit of 10^{-3} mg/mL). SY: Yogurt supplemented with soy protein isolate

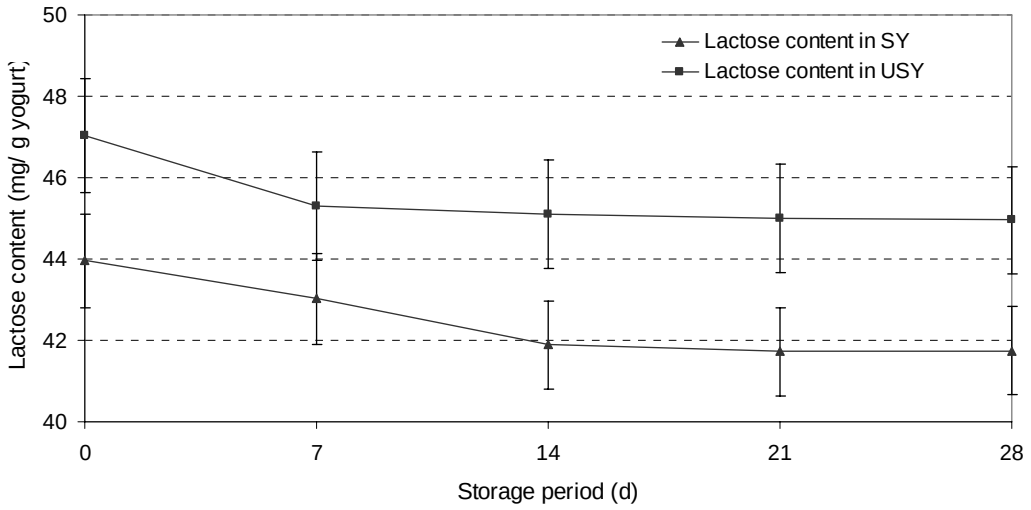


Figure 6.1 Lactose content in the SY and USY (mg/g yogurt) during the storage at 4 ^{o}C

Results are expressed as mean ± standard error (n = 6)

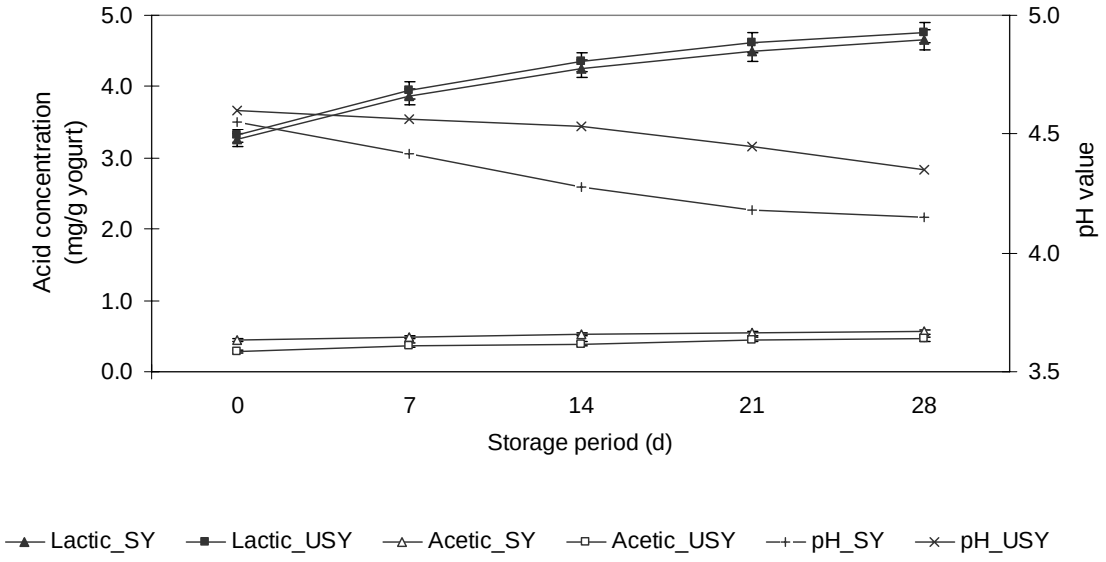


Figure 6.2 Lactic acid and acetic acid concentrations (mg/g yogurt) and pH values of the SY and USY during the storage at 4 ^{o}C

Results are expressed as mean ± standard error (n = 6)

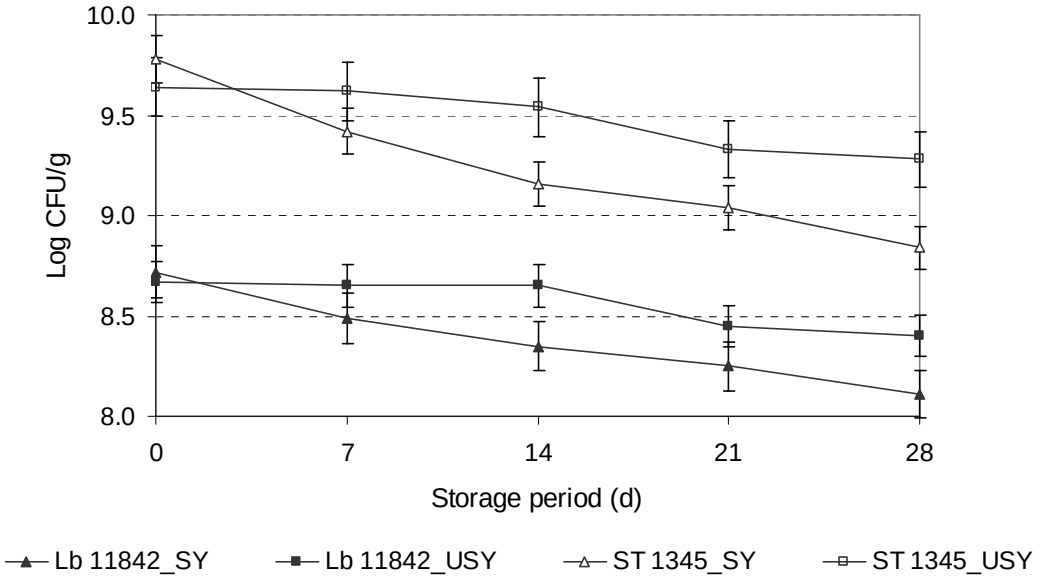
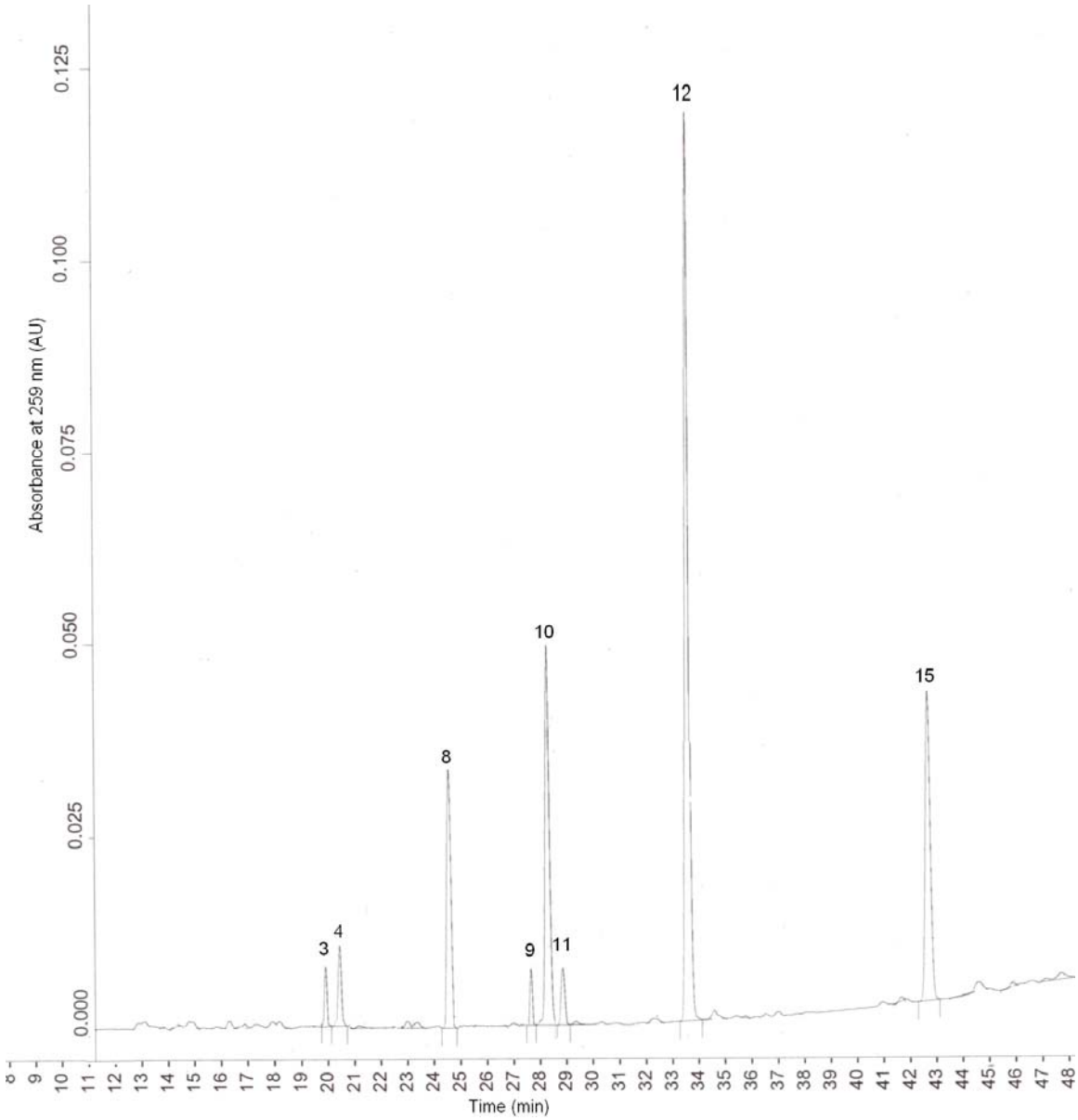


Figure 6.3 Viability of microorganisms (log CFU/g) in the yogurts during 28 d storage at 4 ^{o}C

Results are expressed as mean ± standard error (n = 6)



The peaks are: 3-malonyl daidzin, 4- malonyl glycitin, 8-malonyl genistin, 9- acetyl genistin, 10-daizein, 11-glycitein, 12- genistein and 15-flavone.

Figure 6.4 Chromatogram of isoflavone compounds in SY at 28 day of the storage period at 4 ^{o}C

Chapter 7.0

Summary of significant findings and future research directions

β-Galactosidase was able to hydrolyse the β-glucosidic bond between a β-glycoside and an IA in IG molecule. At 4.0 U/mL of β-galactosidase, up to 77.1% of total IG were hydrolysed in 240 min at 37 ^{o}C. This suggests that β-galactosidase has a flexible structure, its active site is able to reshape by interactions with IG. Generally, β-glucosidase has been claimed to be the only enzyme which is able to hydrolyse isoflavone glycosides to aglycones. Hence, with the evidence of the effective hydrolysis of IG to IA by β-galactosidase, a novel method of production of IA will open as β-galactosidase is more abundant enzyme in traditional microorganisms such as LAB and probiotics.

Supplementation with lactulose (0.5%, w/w) to soymilk enhanced the biotransformation level of IG to IA significantly from 9.6 to 20.6% within 24 h of fermentation by both lactobacilli and bifidobacteria in the 6 strains that were studied including *Lactobacillus acidophilus* 4461, *L. acidophilus* 4962, *L. casei* 290 and *L. casei* 2607, *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099. In particular, *L. acidophilus* 4461 achieved the biotransformation level of 88.8% in soymilk supplemented with lactulose. This is the highest level of the transformation of soy IG has been published. The reason for the enhancing effect of lactulose is possibly due to the enhancing β-galactosidase activity produced by the microorganisms in presence of lactulose in the medium. A significant improvement ($P < 0.05$) in the growth of microorganisms due to the supplementation with lactulose was also observed.

Supplementation with SMP to soymilk increased the biotransformation of IG to IA considerably from 9.7 to 19.0% by the 6 organisms studied. Supplementation with SMP also played a key role in decreasing the pH of the medium. The presence of SPI stimulated the lactose utilisation by 3 – 5 mg/mL, but the effect varied with probiotic organisms. The biotransformation level of IG to IA achieved the highest level of 85% by *L. acidophilus* 4962 and *B. longum* 20099.

Unlike the supplementation with SMP that had the enhancing effects during the entire incubation, while lactulose supplementation only had the enhancing effect after 12h of

incubation by all the six probiotic strains studied. That may be due to a variety of nutritional components in SMP that the probiotic organisms may used immediately.
The enhancing effect of lactulose supplementation was slightly higher than the SMP supplementation on *L. acidophilus* 4461 and *B. animalis* subsp. *lactis* bb12, but slightly lower on the other four probiotic strains.

Finally, the biotransformation and the concentration of IA in yogurt enriched with SPI during the cold storage at 4 ^{o}C have been studied. The supplementation with SPI to yogurt mix had a significant impact on the performance of the yogurt starter such as promoting the metabolism of lactose. The yogurt starter appeared to have a good capability for biotransformation of IG to IA. Within only 8 h of incubation, 72.8% of the total IG was converted to IA, increasing the amount of IA by 11.1 times. Therefore, SY contained a high concentration of the live yogurt starter (8.11-8.84 log CFU/g), and a considerable amount of IA (15.02- 15.51 mg/100 g of freeze-dried sample) and may provide enormous health benefits for human.

Further suggestions relating to study on in soy food and SI are:

- Finding more sources of enzymes which are able to hydrolyse of IG to IA.
- Examining the biotransformation of the two absent isoflavone glycosides in SPI, ononin and sissotrin by probiotic organisms
- Comparing the biotransformation level of probiotic organisms with yogurt starter
- Investigating the biotransformation of IG to IA in soy yogurt fermented by the combination of yogurt starter and probiotic organisms during storage period.

Chapter 8.0

References

Adams, M. R., Golden, D. L., Williams, J. K., Franke, A. A., Register, T. C., & Kaplan, J. R. (2005). Soy protein containing isoflavones reduces the size of atherosclerotic plaques without affecting coronary artery reactivity in adult male monkeys. Journal of Nutrition, 135, 2852-2856.

Adlercreutz, C., Goldin, B., Gorbach, S., Hockerstedt, K., Watanabe, S., Hamalainen, E., Markkanen, M., Makela, T., Wahala, K., & Adlercreutz, T. (1995). Soybean phytoestrogen intake and cancer risk. Journal of Nutrition, 125, 757S-767S.

Albert, A., Altabre, C., Baró, F., Buendía, E., Cabero, A., Cancelo, M. J., Castelo-Branco, C., Chantre, P., Duran, M., Haya, J., Imbert, P., Juliá, D., Lanchares, J. L., Llaneza, P., Manubens, M., Miñano, A., Quereda, F., Ribes, C., & Vázquez, F. (2002). Efficacy and safety of a phytoestrogen preparation derived from Glycine max (L.) Merr in climacteric symptomatology: A multicentric, open, prospective and non-randomized trial. Phytomedicine, 9, 85-92.

Albertazzi, P., Pansini, F., Bonaccorsi, G., Zanotti, L., Forini, E., & Aloysio, D. D. (1998). The effect of dietary soy supplementation on hot flushes. Obstetrics and Gynecology, 91, 6-11.

Allbright, S., Winston, W., & Zappe C. (1999). Data analysis and decision making with Microsoft Excel. Pacific Grove, CA: Duxbury Press. 996p.

Allred, C., Ju, Y., Allred, K., Chang, J., & Helferich, W. (2001). Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein. Carcinogenesis, 22, 1667-1673.

American Association of Cereal Chemist Approved Methods Committee (AACC). (2000). Approved methods of the American Association of Cereal Chemists. 10 th ed. St Paul, Minn: American Association of Cereal Chemists.

Anthony, M. S., Clarkson, T. B., Bullock, B. C., & Wagner, J. D. (1997). Soy protein versus soy phytoestrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys. Arteriosclerosis, Thrombosis, and Vascular Biology, 17, 2524-2531.

Anthony, M. S., Clarkson, T. B., Hughes, C. L., Morgan, T. M., & Burke, G. L. (1996). Soybean isoflavones improve cardiovascular risk factors without affecting the reproductive system of peripubertal rhesus monkeys. Journal of Nutrition, 126, 43-50.

Ariyama, H. (1963). Process for the manufacture of synthetic yogurt from soybean. USA Patent 3,096,177.

Baird, D., Umbach, D., Lansdell, L., Hughes, C., Setchell, K., Weinberg, C., Haney, A., Wilcox, A., & McLachlan, J. (1995). Dietary intervention study to assess estrogenicity of dietary soy among postmenopausal women. Journal of Clinical Endocrinology & Metabolism, 80, 1685-1690.

Barnes, S. (1995). Effect of genistein on *in vitro* and *in vivo* models of cancer. Journal of Nutrition, 125, 777S-783S.

Barnes, S., Coward, L., Kirk, M., & Sfakianos, J. (1998). HPLC-mass spectrometry analysis of isoflavones. Proceedings of the Society for Experimental Biology and Medicine, 217, 254-262.

Barnes, S., Sfakianos, J., Coward, L., & Kirk, M. (1996). Soy isoflavonoids and cancer prevention. Underlying biochemical and pharmacological issues. Advances in Experimental Medicine & Biology, 401, 87-100.

Bosetti, C., Bravi, F., Talamini, R., Parpinel, M., Gnagnarella, P., Negri, E., Montella, M., Lagiou, P., Franceschi, S., & Vecchia, C. L. (2006). Flavonoids and prostate cancer risk: A study in Italy. Nutrition and Cancer, 56, 123-127.

Brzezinki, A., & Debi, A. (1999). Phytoestrogens: the "natural" selective estrogen receptor modulators? European Journal of Obstetrics & Gynecology and Reproductive Biology, 85, 47-51.

Challem, J. (1997). Are soy isoflavones the women's health powerhouse? http://www.thenutritionreporter.com/soy_isoflavones.html, accessed 28 Feb 2009.

Chandan, R. C. (2006). Milk composition, physical and processing characteristics in: Chandan RC, editor. Manufacturing of Yogurt and Fermented Milk. Oxford, UK: Blackwell Publishing Ltd. p 17-40

Chang, H., Churchwell, M., Delclos, K., Newbold, R., & Doerge, D. (2000). Mass spectrometric determination of genistein tissue distribution in diet-exposed Sprague-Dawley rats. Journal of Nutrition, 130, 1963-1970.

Chavez-Servin, J. L., Castellote, A. I., & Lopez-Sabater, M. C. (2004). Analysis of mono- and disaccharides in milk-based formulae by high-performance liquid chromatography with refractive index detection. Journal of Chromatography A, 1043, 211-215.

Chavez-Servin, J. L., Romeu-Nadal, M., Castellote, A. I., & Lopez-Sabater, M. C. (2006). Evolution of free mono and di-saccharide content of milk-based formula powder during storage. Food Chemistry, 97, 103-108.

Chen, Y. M., Ho, S. C., Lam, S. S. H., Ho, S. S. S., & Woo, J. L. F. (2003). 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, 4740-4747.

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

Choi, Y. B., Sim, 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., Choi, I. D., Kwon, G. H., Park, J. Y., Jeong, S. J., Kim, J. S., & Kim, J. H. (2007). Conversion of Isoflavone glucosides to aglycones in soymilk by fermentation with lactic acid bacteria. Journal of Food Science, 72, M39-M44.

Chun, J., Kim, J. S., & Kim, J. H. (2008). Enrichment of isoflavone aglycones in soymilk fermented with single and mixed cultures of *Streptococcus infantarius* 12 and *Weissella* sp. 4. Food Chemistry, 109, 278-284.

Cimino, C. O., Shelnutt, S. R., Ronis, M. J. J., & Badger, T. M. (1999). An LC-MS method to determine concentrations of isoflavones and their sulfate and glucuronide conjugates in urine. Clinica Chimica Acta, 287, 69-82.

Coldham, N., Howells, L., Santi, A., Montesissa, C., Langlais, C., King, L., Macpherson, D., & Sauer, M. (1999). Biotransformation of genistein in the rat: elucidation of metabolite structure by product ion mass fragmentology. Journal of Steroid Biochemistry & Molecular Biology, 70, 169-184.

Coldham, N. G., Darby, C., Hows, M., King, L. J., Zhang, A.-Q., & Sauer, M. J. (2002). Comparative metabolism of genistin by human and rat gut microflora: detection andidentification of the end-products of metabolism. Xenobiotica, 32, 45-62.

Coward, L., Smith, M., Kirk, M., & Barnes, S. (1998). Chemical modification of isoflavones in soy foods during cooking and processing. American Journal of Clinical Nutrition, 68, 1486S-1491S.

Cummings, S., Eckert, S., Kruege, K., Grady, D., Powles, T., Cauley, J., Norton, L., Nickelsen, T., Bjarnason, N., Morrow, M., Lippman, M., Black, D., Glusman, J., Costa, A., & Jordan, V. (1999). The effect of Raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple outcomes of raloxifene evaluation. Journal of the American Medical Association, 281, 2189-2197.

Dave, R. I., & Shah, N. P. (1996). Evaluation of media for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, and bifidobacteria. Journal of Dairy Science, 79, 1529-1536.

Davis, J. N., Kucuk, O., & Sarkar, F. H. (1999). Genistein inhibits NF-kB activation in prostate cancer cells. Nutrition and Cancer, 35, 167-174.

Day, A., Dupond, M., Ridley, S., Rhodes, M., Rhodes, M., Morgan, M., & William, G. (1998). Deglycosylation of flavonoid glycosides by human small intestine and liver β-glucosidase activity. FEBS Letters, 436, 71-75.

Delmonte, P., Perry, J., & Rader, J. I. (2006). Determination of isoflavones in dietary supplements containing soy, red clover and kudzu: extraction followed by basic or acid hydrolysis. Journal of Chromatography A, 1107, 59-69.

Desai, A. R., Powell, I. B., & Shah, N. P. (2004). Survival and activity of probiotic lactobacilli in skim milk containing prebiotics. Journal of Food Science, 69, FMS57-FMS60.

Divi, R., Chang, H., & Doerge, D. (1997). Anti-thyroid isoflavones from soybean: isolation, characterization, and mechanisms of action. Biochemical Pharmacology, 54, 1087-1096.

Dixon, R. A. (2004). Phytoestrogens. Annual Review of Plant Biology, 55, 225-261.

Doerge, D. R., & Sheehan, D. M. (2002). Goitrogenic and estrogenic activity of soy isoflavones. Environmental Health Perspectives, 110 (suppl. 3), 349-353.

Donkor, O. N., Hendrickson, A., Vasiljevic, T., & Shah, N. P. (2005). Probiotic strains as starter cultures improve angiotensin-converting enzyme inhibitory activity in soy yogurt. Journal of Food Science, 70, M375-M381.

Donkor, O. N., Nilmini, S. L. I., Stolic, P., Vasiljevic, T., & Shah, N. P. (2007). Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. International Dairy Journal, 17, 657-665.

Donkor, O. N., & Shah, N. P. (2008). Production of β-glucosidase and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus casei* in soymilk. Journal of Food Science, 73, M15-M20.

Dubey, U. K., & Mistry, V. V. (1996). Effect of bifidogenic factors on growth characteristics of bifidobacteria in infant formulas. Journal Dairy Science, 79, 1156-1163.

FAO/WHO. (2001). Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria.

Farnworth, E., Mainville, I., Desjardins, M., Gardner, N., Fliss, I., & Champagne, C. (2007). Growth of probiotic bacteria and bifidobacteria in a soy yogurt formulation. International Journal of Food Microbiology, 116(174-181).

Fernandez-Garcia, E., & McGregor, J. U. (1994). Determination of organic acids during the fermentation and cold storage of yogurt. Journal of Dairy Science, 77, 2934-2939.

File, S. E., Jarrett, N., Fluck, E., Duffy, R., Casey, K., & Wiseman, H. (2001). Eating soya improves human memory. Psychopharmacology, 157, 430-436.

Fischer, E. (1894). Einfluss der configuration auf die wirkung der enzyme. Berichte 27, 2985–2993.

Frankenfeld, C. L., Atkinson, C., Thomas, W. K., Gonzalez, A., Jokela, T., Wahala, K., Schwartz, S. M., Li, S. S., & Lampe, J. W. (2005). High concordance of daidzein-metabolizing phenotypes in individuals measured 1 to 3 years apart. The British Journal of Nutrition, 94, 873-876.

Frye, C. P. (2006). Regulation for product standards and labelling in: Chandan RC, editor. Manufacturing Yogurt and Fermented Milk. Oxford, UK: Blackwell Publishing Ltd. p 57-72

Fujioka, M., Uehara, M., Wu, J., Adlercreutz, H., Suzuki, K., Kanazawa, K., Takeda, K., Yamada, K., & Ishimi, Y. (2004). Equol, a metabolite of daidzein, inhibits bone loss in ovariectomized mice. Journal of Nutrition, 134, 2623–2627.

Garbutt, J. (1997). Essentials of Food Microbiology. London, UK: Arnold.

Gomes, A. M. P., Malcata, F. X. & Klaver, F. A. M. (1998). Growth enhancement of *Bifidobacterium lactis* Bo and *Lactobacillus acidophilus* Ki by milk hydrolyzates. Journal of Dairy Science, 81, 2817-2825.

Gonzales, A. S. P., Naranjo, G. B., Malec, L. S., & Vigo, M. S. (2003). Available lysine, protein digestibility and lactulose in commercial infant formulas. International Dairy Journal, 13, 95-99.

Ghosh & Fenner (1999). Improved Method for Gas Chromatographic Analysis of Genistein and Daidzein from Soybean (Glycine max) Seeds, Journal of Agricultural and Food Chemistry, 47, 3455-3456.

Griffith, A. P., & Collison, M. W (2001). Improved methods for the extraction and analysis of isoflavones from soy- containing foods and nutritional supplements by reversed-phase high-performance liquid chromatography and liquid chromatography -mass spectrometry. Journal of Chromatography A 913, 397-413.

Gibson, G., & Roberfroid, M. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. Journal of Nutrition, 125, 1401-1412

Hamilton-Reeves, J. M., Rebello, S. A., Thomas, W., Kurzer, M. S., & Slaton, J. W. (2008). Effects of soy protein isolate consumption on prostate cancer biomarkers in men with HGPIN, ASAP, and low-grade prostate cancer. Nutrition and Cancer, 60, 7-13.

Han, K., Soares, J. J., Haidar, M., Lima, G. D., & Baracat, E. (2002). Benefits of soy isoflavone therapeutic regimen on menopausal symptoms. Obstetrics and Gynecology, 99, 389-394.

Higdon, J. (2006). Soy Isoflavone: Linus Pauling Institute, Oregon State University, http://lpi.oregonstate.edu/infocenter/phytochemicals/soyiso/. Accessed 25 April 2009

Hofman, M., & Thonart, P. (2001). Engineering and Manufacturing for Biotechnology. Dordrecht, the Netherlands: Kluwer Academic Publishers.

Holzapfel, W. H., & Wood, B. J. B. (eds.). (1998). The genera of lactic acid bacteria (1st ed.). Glasgow, UK. London Blackie Academic & Professional.

Horiia, K., Adachib, T., Matsudab, T., Tanakac, T., Saharad, H., Shibasakie, S., Oginob, C., Hatad, Y., Uedaf, M., & Kondob, A. (2009). Improvement of isoflavone aglycones production using β-glucosidase secretory produced in recombinant *Aspergillus oryzae*. Journal of Molecular Catalysis B: Enzymatic, 59, 297-301.

Huber, R. E., Hurlburt, K. L., & Turner, C. L. (1981). The anomeric specificity of β-galactosidase and lac permease from *Escherichia coli*. Canadian Journal of Biochemistry. 59, 100–105.

Hughes, I., Aggett, P., Ariyanayagam, S., Carthew, P., Chipman, J. K., Jackson, P., Joffe, M., Kimber, I., Lunec, J., Piersma, A., Rowland, I. R., Rushton, L., Salfield, J., Smith, A. G., Stanley, L., Strobel, S., Timbrell, J. A., Tucker, M., Benford, D., Butler, K., Gott, D., Mulholland, C. A., Tahourdin, C., Thatcher, N., Maycock, B., Ball, N., Moizer, K., & Sivapathasundaram, S. (2003). Phytoestrogens and Health. London: Food Standards Agency.

Hussain, M., Banerjee, M., Sarkar, F. H., Djuric, Z., Pollak, M. N., Doerge, D., Fontana, J., Chinni, S., Davis, J., Jeffrey Forman, Wood, D. P., & Kucuk, O. (2003). Soy isoflavones in the treatment of prostate cancer. Nutrition and Cancer, 47, 111-117.

Ikeda, Y., Iki, M., Morita, A., Kajita, E., Kagamimori, S., Kagawa, Y., & Yoneshimayy, H. (2006). Intake of fermented soybeans, natto, is associated with reduced bone loss in postmenopausal women: Japanese population-based osteoporosis (JPOS) study. Journal of Nutrition, 136, 1323–1328.

Ismail, B., & Hayes, K. (2005). β-glycosidase activity toward different glycosidic forms of isoflavones. Journal of Agricultural and Food Chemistry, 53, 4918-4924.

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

Jeri, A. R. (2002). The use of an isoflavone supplement to relieve hot flushes. Female Patient, 27, 35-37.

Joannoua, G. E., Kellyb, G. E., Reedera, A. Y., Waringb, M., & Nelsona, C. (1995). A urinary profile study of dietary phytoestrogens. The identification and mode of

metabolism of new isoflavonoids. Journal of Steroid Biochemistry & Molecular Biology, 54, 167-184.

Johnson, D. W. (1975). Use of soy products in dairy product replacement. Journal of the American Oil Chemist's Society, 52, 270A - 271A.

Juskiewicz, J., & Zdunczyk, Z. (2002). Lactulose-induced diarrhoea in rats: effects on caecal development and activities of microbial enzymes. Comparative Biochemistry and Physiology Part A, 133, 411-417.

Kamaly, K. M. (1997). Bifidobacteria fermentation of soy bean milk. Food Research International, 30, 675-682.

Kelly, G., Nelson, C., Waring, M., Joannou, G., & Reeder, A. (1993). Metabolites of dietary (soya) isoflavones in human urine. Clinica Chimica Acta, 223, 9-22.

Kim, M. K., Kim, J. H., Nam, S. J., Ryu, S., & Kong, G. (2008). Dietary intake of soy protein and tofu in association with breast cancer risk based on a case-control study. Nutrition and Cancer, 60, 568–576.

King, R. A. (1998). Daidzein conjugates are more bioavailable than genistein conjugates in rat. American Journal of Clinical Nutrition, 68, 1496S-1499S.

King, R. A., & Bignell, C. M. (2000). Concentration of isoflavone phytoestrogens and their glucosides in Australian soya beans and soya foods. Australian Journal of Nutrition and Dietetics, 57(2), 70-78.

King, R., & Bursill, D. (1998). Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single meal in humans. American Journal of Clinical Nutrition, 67, 867-872.

Klejdus, B., Mikelova, R., Petrlova, J., Potesil, D., Adam, V., Stiborova, M., Hodek, P., Vacek, J., Kizek, R., & Kuban, V. (2005). Evaluation of isoflavone aglycon and glycoside distribution in soy plants and soybeans by fast column high-performance liquid chromatography coupled with a diode -array detector. Journal of Agricultural and Food Chemistry. 53, 5848–5852.

Kneifel, W., Rajal, A., & Kulbe, K. D. (2000). *In vitro* growth behaviour of probiotic bacteria in culture media with carbohydrates of prebiotic. Microbial Ecology in Health and Disease, 12, 27-34.

Knight, D., Eden, J., Huang, J., & Waring, M. (1998). Isoflavone content of infant foods and formulas. Journal of Paediatrics and Child Health, 34, 135-138.

Knight, D., Wall, P. L., & Eden, J. (1996). A review of phytoestrogens and their effects in relation to menopausal symptoms. Australian Journal of Nutrition and Dietetics, 53, 5-11.

Kontula, P., Suihko, M.-L., Wright, A. V., & Mattila-Sandholm, T. (1999). The effect of lactose derivatives on intestinal lactic acid bacteria. Journal of Dairy Science, 82, 249-256.

Kurzer, M. S. (2002). Hormonal effects of soy in premenopausal women and men. Journal of Nutrition, 132, 570S-573S.

Li, Y., Upadhyay, S., Bhuiyan, M., & Sarkar, F. (1999). Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein. Oncogene, 18, 3166-3172.

Lichtenstein, A. H. (1998). Soy Protein, isoflavones and cardiovascular disease risk. Journal of Nutrition, 128, 1589–1592.

Liggins, J., Bluck, L. J. C., Runswick, S., Atkinson, C., Coward, W. A., & Bingham, S. A. (2000a). Daidzein and genistein content of fruit and nuts. Journal of Nutritional Biochemistry, 11, 326-331.

Liggins, J., Bluck, L. J. C., Runswick, S., Atkinson, C., Coward, W. A., & Bingham, S. A. (2000b). Daidzein and genistein contents of vegetables. British Journal of Nutrition, 84, 717-725.

Liu, K. (2004). Soybean as functional foods and ingredients: AOCS Press, Champaign, Illinois, USA.

Lu, J. J., & Anderson, K. (1998). Sex and long term soy diets affect the metabolism and excretion of soy isoflavones in humans. American Journal of Clinical Nutrition, 68, 1500S-1504S.

Marotti, I., Bonetti, A., Biavati, B., Catizone, P., & Dinelli, G. (2007). Biotransformation of common bean (*Phaseolus vulgaris L.*) flavonoid glycosides by *Bifidobacterium* species from human intestinal origin. Journal of Agricultural and Food Chemistry, 55, 3913-3919.

Malnig, A., & Brown, J. (2007). Soy: How safe is it? Health Dilemmas, April, 24-26.

Mathias, K., Ismail, B., Corvalan, C. M., & Hayes, K. D. (2006). Heat and pH effects on the conjugated form of genistin and daidzin isoflavones. Journal of Agricultural and Food Chemistry, 54, 7495-7502.

Mazur, W. M., Duke, J. A., Wahala, K. S. R., & Adlercreutz, H. (1998). Isoflavonoids and lignans in legumes:nutritional and health aspects in humans. Journal of Nutritional Biochemistry, 9, 193-200.

Medline Plus. (2008). Menopausal. http://www.nlm.nih.gov/medlineplus/ency/article/000894.htm. Accessed 28 Feb 2009.

Messina, V., & Messina, M. (2003). Is it safe to eat soy? http://www.veganhealth.org/articles/soymessina, Accessed 20 March 2009.

Mitchell, J., Cawood, E., Kinniburgh, D., Provan, A., Collins, A., & Irvine, D. (2001). Effect of a phytoestrogen food supplement on reproductive health in normal males. Clinical Science, 100, 613-618.

Moscone, D., Bernardo, R.A., Marconi, E., Aminec, A., & Palleschia G. (1999). Rapid determination of lactulose in milk by microdialysis and biosensors. The Analyst 124, 325–329.

Nagata, C. (2000). Ecological study of the association between soy product intake and mortality from cancer and heart disease in Japan. International Journal of Epidemiology, 29, 832-836.

Nagata, C., Takatsuka, N., Kawakami, N., & Shimizu, H. (2001). Soy product intake and hot flashes in Japanese women: Results from a community-based prospective study. American Journal of Epidemiology, 153, 790-793.

Nakamura Y., Kaihara A., Yoshii K., Tsumura Y., Ishimitsu S., & Tonogai Y. (2001). Content and composition of isoflavonoids in mature or immature beans and bean sprouts consumed in Japan. Journal of Health Science, 47, 394-406.

Nestel, P. (2002). Role of soy protein in cholesterol-lowering: How good is it? Arteriosclerosis, Thrombosis, and Vascular Biology, 22, 1743-1744.

Nestel, P. J., Yamashita, T., Sasahara, T., Pomeroy, S., Dart, A., Komesaroff, P., Owen, A., & Abbey, M. (1997). Soy isoflavones improve systemic arterial compliance but not plasma lipids in menopausal and perimenopausal women. Arteriosclerosis, Thrombosis, and Vascular Biology, 17, 3392-3398.

Nikander, E., Metsa, M., Heikkila, Ylikorkala, O., & Tiitinen, A. (2004). Effects of phytoestrogens on bone turnover in postmenopausal women with a history of breast cancer. Journal of Clinical Endocrinology & Metabolism, 89, 1207-1212.

Nutrition Data. (2007). Soy Protein Isolate: http://www.nutritiondata.com/facts-C00001-01c218n.html. Accessed 15 March 2007.

Otieno, D.O., Ashton, J. F., & Shah, N. P. (2005). Stability of β-glucosidase activity processing by *Bifidobacterium* and *Lactobacillus* spp. in fermented soymilk during processing and storage. Journal of Food Science, 70, 236-41.

Otieno, D. O., Ashton, J. F., & Shah, N. P. (2006a). Evaluation of enzymic potential for biotransformation of isoflavone phytoestrogen in soymilk by *Bifidobaterium 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, 1182-1191.

Otieno, D. O., Ashton, J. F., & Shah, N. P. (2007). Isoflavone phytoestrogen degradation in fermented soymilk with selected β-glucosidase producing *L. acidophilus* strains during storage at different temperatures. International Journal of Food Microbiology, 115, 79-88.

Ouwehan, A. C., & Salminen, S. J. (1998). Health effects of probiotics and culture-containing dairy products in humans. Paper presented at the International Dairy Federation (IDF), Brusell.

Pham, T. T., & Shah, N. P. (2007). Biotransformation of isoflavone glycosides by *Bifidobacterium animalis* in soymilk supplemented with skim milk powder. Journal of Food Science, 72(8), M316 -M324.

Pham, T. T., & Shah, N. P. (2008a). Fermentation of reconstituted skim milk supplemented with soy protein isolate by probiotic organisms. Journal of Food Science, 73, M62-M66.

Pham, T. T., & Shah, N. P. (2008b). Effect of lactulose on biotransformation of isoflavone glycosides to aglycones in soymilk by lactobacilli. Journal of Food Science, 73, M158-M165.

Pham, T. T., & Shah, N. P. (2008c). Skim milk powder supplementation affects lactose utilisation, microbial survival and biotransformation of isoflavone glycosides to

isoflavone aglycones in soymilk by *Lactobacillus*. Food Microbiology, 25, 653-661.

Pham, T. T., & Shah, N. P. (2008d). Effect of lactulose supplementation on the growth of bifidobacteria and biotransformation of isoflavone glycosides to isoflavone aglycones in soymilk. Journal of Agricultural and Food Chemistry, 56, 4703-4709.

Pham, T. T., & Shah, N. P. (2009a). Hydrolysis of isoflavone glycosides in soymilk by β-galactosidase and β-glucosidase. Journal of Food Biochemistry, 33, 38-60.

Pham, T. T., & Shah, N. P. (2009b). Performance of starter in yogurt supplemented with soy protein isolate and biotransformation of isoflavones during storage period. Journal of Food Science, 74, M190-M195.

Pham, T. T., & Shah, N. P. (2009c). Effects of skim milk powder supplementation to soy yogurts on biotransformation of isoflavone glycosides to biologically active forms during storage. Proceeding of the International Conference on Agricultural, Food and Nutrition Sciences, Dubai, UAE, ISSN: 2070-3740.

Picherit, C., Coxam, V., Bennetau-Pelissero, C., Kati-Coulibaly, S., Davicco, M.-J., Lebecque, P., & Barlet, J. P. (2000). Daidzein is more efficient than genistein in preventing ovariectomy-induced bone loss in rats. Journal of Nutrition, 130, 1675-1681.

Pike, A., Brzozowski, A., Hubbard, R., Bonn, T., Thorsell, A. G., Engström, O., Ljunggren, J., Gustafsson, J. Å., & Carlquist, M. (1999). Structure of the ligand-binding domain of oestrogen receptor β in the presence of a partial agonist and a full antagonist. European Molecular Biology Organization Journal, 18, 4608-4618

Piskula, M. K., Yamakoshi, J., & Iwai, Y. (1999). Daidzein and genistein but not their glucosides are absorbed from the rat stomach. FEBS Letters, 447, 287-291.

Poch, M., & Bezkorovainy, A. (1988). Growth-enhancing supplements for various species of the genus *Bifidobacterium*. Journal of Dairy Science 71, 3214-3221.

Riaz, M. N. (2006). Soy application in food. Boca Raton: Taylor & Francis Group.

Richelle, M., Pridmore-Merlen, S., Bodenstab, S., Enslen, M., & Offord, E. A. (2002). Hydrolysis of isoflavone glycosides to aglycones by β-glycosidase does not alter plasma and urine isoflavone pharmacokinetics in postmenopausal women. Journal of Nutrition, 132, 2587-2592.

Rowland, I., Wiseman, H., Sanders, T., Adlercreutz, H., & Bowey, E. (1999). Metabolism of oestrogens and phytoestrogens: role of the gut microflora. Biochemical Society Transactions, 27, 304-308.

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

Saitoh, S., Sato, T., Harada, H., & Matsuda, T. (2004). Biotransformation of soy isoflavone-glycosides in laying hens: intestinal absorption and preferential accumulation into egg yolk of equol, a more estrogenic metabolite of daidzein. Biochimica et Biophysica Acta (G). General subjects, 1674, 122- 130.

Salminen, S., & Salminen, E. (1997). Lactulose, lactic acid bacteria, intestinal microecology and mucosal protection. Scandinavian Journal of Gastroenterology. Supplement, 222, 45-48.

Sanitarium Health Food Company. (2004). The soy story: Soy and Heart Disease. http://www.soyfacts.com.au/cancer/oestrogen.html. Accessed 3 March 2009.

Sarkar, F. H., & Li, Y. (2003). Soy isoflavones and cancer prevention. Cancer Investigation, 21, 744-757.

Sartippour, M. R., Rao, J. Y., Apple, S., Wu, D., Henning, S., Wang, H., Elashoff, R., Rubio, R., Heber, D., & Brooks, M. N. (2004). A pilot clinical study of short-term isoflavone supplements in breast cancer patients. Nutrition and Cancer, 49, 59–65.

Setchell, K. D. (1998). Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. American Journal of Clinical Nutrition, 68(6), 1333-1346.

Setchell, K. D., Brown, N. M., Zimmer-Nechemias, L., Brashear, W. T., Wolfe, B. E., Kirschner, A. S., & Heubi, J. E. (2002). Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. American Journal of Clinical Nutrition, 76, 447-453.

Setchell, K. D., Clerici, C., Lephart, E. D., Cole, S. J., Heenan, C., Castellani, D., Wolfe, B. E., Nechemias-Zimmer, L., Brown, N. M., Lund, T. D., & Handa, R. J., & Heubi, J. E. (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. (2001). Soy isoflavones- Benefits and risks from nature's selective estrogen receptor modulators (SRRMs). Journal of the American College of Nutrition, 20(5), 354-365.

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

Setchell, K. D. R., & Cassidy, A. (1999). Dietary isoflavones: biological effects and relevance to human health. Journal of Nutrition, 139(3), 758-767.

Shah, N. P. (1993). Effectiveness of dairy products in alleviation of lactose intolerance. Food Australia. 45, 268–271.

Shah, N. P. (2006). Probiotics and fermented milks. In: Chandan RC. Manufacturing yoghurt and fermented milks. Iowa, USA: Blackwell Publishing Professional. p341-54.

Shah, N. P., & Jelen, P. (1990). Survival of lactic acid bacteria and their lactases under acidic conditions. Journal of Food Science 55, 506-509.

Shelef, L. A., Bahnmiller, K. R., Zemel, M. B., & Monte, L. M. (1988). Fermentation of soymilk with commercial freeze-dried starter lactic cultures. Journal of Food Processing & Preservation, 12, 187-195.

Slavin, J., Karr, S., Hutchins, A., & Lampe, J. (1998). Influence of soybean processing, habitual diet, and soy dose on urinary isoflavonoid excretion. American Journal of Clinical Nutrition, 68 (suppl), 1492S-1495S.

Slavine, J. L. (1996). Phytoestrogens in breast milk- Another advantage of breast - feeding? Clinical Chemistry, 42, 841-842.

Snyder, H. E., & Kwon, T. W. (1987). Soybean utilization. New York: Van Nostrand Reinhold Company Inc. 346p.

Somekawa, Y., Chiguchi, M., Ishibashi, T., & Aso, T. (2001). Soy intake related to menopausal symptoms, serum lipids and bone mineral density in postmenopausal Japanese women. Obstetrics and Gynecology, 97, 109-115.

Sounga, D. Y., Khalila, D. A., Arquitta, A. B., Smitha, B. J., Hammonda, L. J., Drokea, E. A., Lucasa, E. A., Devareddya, L., & Arjmandi, B. H. (2004). Soy isoflavones prevent the ovarian hormone deficiency-associated rise in leukocytes in rats. Phytomedicine, 11, 303-308.

Soyfoods Association of North America. (2007). Soy Protein Isolate http://www.soyfoods.org/products/soy-fact-sheets/soy-protein-isolate/ (Accessed 15 August 2007)

Swanson, C., Mao, B., Li, J., Lubin, J., Yao, S., Wang, J., Cai, S., Hou, Y., Lou, Q., & Blot, W. (1992). Dietary determinants of lung-cancer risk: results from a case-control study in Yunnan Province. International Journal of Cancer, 50, 876-880.

Sugano, M. (2005). Soy in Health and Disease Prevention: Hoboken : CRC Press. 328p.

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

Tsangalis, D., & Shah, N. P. (2004) Development of an isoflavone aglycone-enriched soymilk using soy germ, soy protein isolate and bifidobacteria. Food Research International, 37, 301-312.

Tsunoda, N., Pomeroy, S., & Nestel, P. (2002). Absorption in humans of isoflavones from soy and red clover is similar. Journal of Nutrition, 132, 2199-2201.

Uehara, M., Ohta, A., Sakai, K., Suzuki, K., Watanabe, S., & Adlercreutz, H. (2001). Dietary fructo-oligosaccharides modify intestinal bioavailability of a single dose of genistein and daidzein and affect their urinary excretion and kinetics in blood of rats. Journal of Nutrition, 131, 787-795.

Utkina, E. A., Antoshina, S. V., Selishcheva, A. A., Sorokoumova, G. M., Rogozhkina, E. A., & Shvets, V. I. (2004). Isoflavones daidzein and genistein: Preparation by acid hydrolysis of their glycosides and the effect on phospholipid peroxidation. Russian Journal of Bio-organic Chemistry, 30, 385-390.

Van-Patten, C., Olivotto, I., Chambers, G., Gelmon, K., Hislop, T., Templeton, E., Wattie, A., & Prior, J. C. (2002). Effect of soy phytoestrogens on hot flashes in postmenopausal women with breast cancer: a randomized, controlled clinical trial Journal of Clinical Oncology, 20, 1449-1455.

Vedamuthu, E. R. (2006). Stater cultures for yogurt and fermented milks. In: Chandan RC, editor. Manufacturing Yogurt and Fermented Milk. Oxford: Blackwell Publishing Ltd. p 89-116

Vranova, D. (2005). Quantification of soy isoflavones in meat products by HPLC. Scripta Medica (Brno), 78, 235-242.

Wagner, J. D., Schwenke, D. C., Greaves, K. A., Zhang, L., Anthony, M. S., Blair, R. M., Shadoan, M. K., & Williams, J. K. (2003). Soy protein with isoflavones, but not an isoflavone-rich supplement, improves arterial low-density lipoprotein metabolism and atherogenesis. Arteriosclerosis, Thrombosis, and Vascular Biology, 23, 2241-2246.

Walter, E. D. (1941). Genistein (an isoflavone glucoside) and its aglucone, genistein, from soybeans. Journal of the American Oil Chemists' Society, 63, 3273–3276.

Wang, H., & Murphy, P. (1994). Isoflavone content in commercial soybean foods. Journal of Agricultural and Food Chemistry, 42, 1666-1673.

Wang, H. J., & Murphy, P. A. (1996). Mass balance study of isoflavones during soybean processing. Journal of Agricultural and Food Chemistry, 44, 2377-83

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

Wei, Q. K., Chen, T. R., & Chen, J. T. (2007). Using of *Lactobacillus* and *Bifidobacterium* to product the isoflavone aglycones in fermented soymilk. International Journal of Food Microbiology, 117, 120-124.

White, L. R., Petrovitch, H., Ross, W., Masaki, K., Hardman, J., Nelson, J., Davis, D., & Markesbery, W. (2000). Brain aging and midlife tofu consumption. Journal of the American College of Nutrition, 19, 242–255.

Women's Health Initiative. (2004). Women's Health Initiative HRT Study: http://www.menopauseinsight.com/hormone-replacement/womens-health-initiative-hrt-study.aspx.

Wu, A., Ziegler, R., Horn-Ross, P., Nomura, A., West, D., Kolonel, L., Rosenthal, J., Hoover, R., & Pike, M. (1996). Tofu and risk of breast cancer in Asian-Americans. Cancer Epidemiology Biomarkers & Prevention, 5, 901-906.

Xiang, H., Schevzov, G., Gunning, P., Williams, H. M., & Silink, M. (2002). A comparative study of growth-inhibitory effects of isoflavones and their

metabolites on human breast and prostate cancer cell lines. Nutrition and Cancer, 42, 224-232.

Xu, X., Harris, K. S., Wang, H. J., Murphy, P. A., & Hendrich, S. (1995). Bioavailability of soybean isoflavones depends on gut microflora in women. Journal of Nutrition, 125, 2307-2315.

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

Ye, Y. B., Tang, X. Y., Verbruggen, M. A., & Su, Y. X. (2006). Soy isoflavones attenuate bone loss in early postmenopausal Chinese women: A single-blind randomized, placebo-controlled trial. European Journal of Nutrition, 45, 327–334.

Zhang, M., Xie, X., Lee, A. H., & Binns, C. W. (2004). Soy and isoflavone intake are associated with reduced risk of ovarian cancer in southeast China. Nutrition and Cancer, 49, 125-130.

Zonis, S. 2007. What is probiotic food? http://www.thenibble.com/reviews/nutri/probiotic-food.asp (Accessed: 19 March 2008).