

**STABILITY OF BIOACTIVE ISOFLAVONES AND GLYCOLYTIC ENZYMES
PRODUCED BY PROBIOTIC BACTERIA IN SOY BASED FOOD DURING
PROCESSING AND STORAGE**

A thesis submitted for the degree of Doctor of Philosophy

By

Daniel Obed Otieno

BSc Hons (Food Sci Tech), JKUAT, Kenya; MSc (Food Tech), UNSW, Sydney, Australia

2007

Victoria University

School of Molecular Sciences

Faculty of Science, Engineering and Technology

Werribee,

Australia

This thesis, being my initial meaningful contribution to the body of scientific knowledge, is dedicated to my beloved mother, Mrs. Esther Were Owuor, for her immense sacrifices, encouragement and prayers throughout my life. Her fortitude, unshakeable determination and resolve to raise her 10 children especially after the demise of my hero teacher-father are a great example and inspiration for which I am deeply grateful. The moral support and especially the prayers given by my siblings and the larger family members made this endeavour possible and worthwhile.

The genuine love, understanding and encouragement provided by my dear wife Gladys and my beautiful little girls Kimberly (5) and Talia (3) are forever appreciated.

Abstract

Micro-organisms possess endogenous enzymes, however, the stability of these enzymes during storage in soymilk has not been studied. β -glucosidase is an important enzyme that could be used in the bioconversion of the predominant soy isoflavone glycosides to their bioactive aglycone forms. Fifteen probiotic micro-organisms that included *Bifidobacterium* sp., *Lactobacillus acidophilus* and *Lactobacillus casei* were screened for β -glucosidase activity using p -nitrophenyl- β -D-glucopyranoside as a substrate. Six strains were selected on the basis of β -glucosidase activity produced during fermentation of soymilk. The stability of the enzyme activity was assessed during incubation for up to 48 h and during storage for 8 weeks at frozen (-80°C), refrigerated (4°C), room (25°C) and incubation (37°C) temperatures. *L. casei* strains showed the highest β -glucosidase activity after 24 h of incubation followed by *L. acidophilus* strains, while *Bifidobacterium* strains showed least activity. However, β -glucosidase from *Bifidobacterium animalis* BB12 showed the best stability during the 48 h fermentation. Lower storage temperatures (-80°C and 4°C) showed significantly higher ($P < 0.05$) β -glucosidase activity and better stability than that at higher temperatures (25°C and 37°C). The stability of β -glucosidase from these microorganisms should be considered for enzymic biotransformation during storage, of isoflavone β -glycosides to bioactive isoflavone aglycone forms with potential health benefits.

Three strains of *L. acidophilus*, two strains of *L. casei* and one strain of *Bifidobacterium* were screened for β -glucosidase activity using p -nitrophenyl- β -D-glucopyranoside as a substrate and their potential for the breakdown of isoflavone glycosides to the biologically active aglycones in soymilk. Isoflavones quantification with HPLC and β -glucosidase activity was performed after 0, 12, 24, 36, and 48 h of incubation in soymilk at 37°C . All 6 microorganisms produced β -glucosidase, which hydrolysed the predominant isoflavone β -glycosides. There was a significant increase in the concentration of isoflavone aglycones and a subsequent decrease ($P < 0.05$) in the

concentration of isoflavone glycosides in fermented soymilk. Based on the concentration of isoflavones during peak β -glucosidase activity, the hydrolytic potential was evaluated. *L. acidophilus* 4461 had the highest aglycone concentration of 76.9% after 24 h of incubation, up from 8% in unfermented soymilk (at 0 h). It also had the best isoflavone hydrolytic index of 2.01, signifying its relative importance in altering the biological activity of soymilk.

Soymilk fermented containing soy isoflavones with *B. animalis* Bb12 was stored at various temperatures (-80°C, 4°C, 25°C and 37°C) for 8 weeks and the concentration of isoflavones determined weekly using RP-HPLC. The first order kinetic model was used to assess the degradation of each isoflavone isomer at each storage temperature. During storage at various temperatures, concentrations of individual isoflavone isomers appeared to be significantly stable ($P < 0.01$). Interestingly, the aglycones showed much smaller degradation constants as compared to the glycosides at all the storage temperatures. Genistein and daidzein were much more stable than glycitein and had almost similar degradation pattern, despite differences in their concentrations in the fermented soymilk. It was, however, observed that 4°C was the most suitable storage temperature for the product as there was a minimal degradation of bioactive isoflavone aglycones.

Three selected *L. acidophilus* strains were used in the fermentation of soymilk and then stored separately at various temperatures (-80°C, 4°C, 25°C and 37°C) for 8 weeks and the concentration of isoflavones determined weekly using RP-HPLC with diode array *uv* visible detector. The decreasing concentration of isoflavones in soymilk during storage due to degradation was found to fit first order kinetic model. Isoflavone aglycones as well as isoflavone glycosides largely appeared to be stable during storage ($P < 0.01$). Interestingly, the aglycone forms showed much smaller degradation as compared to glycoside forms at all the storage temperatures. Of the isoflavone aglycones, daidzein was most stable followed by genistein, while glycitein was least stable. Isoflavone aglycones such as glycitein, daidzein and genistein showed

smaller degradation constants in fermented soymilk at lower storage temperatures (-80°C and 4°C) and higher degradation constants at higher storage temperatures (25°C and 37°C) with each strain. In contrast, glycosides glycitin and daidzin showed higher degradation at lower storage temperatures (-80°C and 4°C) and lower degradation at higher storage temperatures (25°C and 37°C). Storage temperature was therefore found to be very important in regulating the rate of degradation of soy isoflavones in fermented soymilk.

The degradation of each isoflavone compound in soymilk fermented with 2 *Lactobacillus casei* strains and stored at various storage temperatures (-80°C, 4°C, 25°C and 37°C) was evaluated and again found to fit the first order kinetic model. All isoflavone compounds in the soymilk appeared to be generally stable during storage ($P < 0.01$) at all storage temperatures. Aglycone forms however, had smaller degradation constants compared to glycosides at all storage temperature in the presence of each of the micro-organisms. Specifically, aglycones showed a unique trend of smaller degradation at lower storage temperatures (-80°C and 4°C) than at higher temperatures (25°C and 37°C). Glycoside genistin was least stable at all storage temperatures compared to other isoflavones, while aglycone daidzein was the most stable. *L. casei* 2607 in fermented soymilk stored at 4°C after 8 weeks gave the least degradation for daidzein of a mere 3.78% loss from 9.53 to 9.17 ng/ μ L. *L. casei* 2607 showed greater hydrolytic potential than *L. casei* ASCC 290 as denoted by higher degradation of isoflavone glycosides in fermented soymilk at lower storage temperatures. The optimum storage temperature offering least degradation of bioactive isoflavone aglycones in fermented soymilk was found to be 4°C.

Liquid chromatography coupled with positive electro spray ionisation tandem mass spectrometry (MS/MS) and diode array detection was used for the quantitation and characterisation of isoflavones in fermented and unfermented soymilk made from soy protein isolate SUPRO 590.

Bifidobacterium animalis ssp. *lactis* Bb12 was used for the fermentation of soymilk. The isoflavones were found to produce characteristic radical ions as well as molecules of H₂O, CO₂, a sugar unit, and an alcohol through collision-induced fragmentation. Product ion fragments revealed unique fragmentation pathways for each isoflavone compound. Characteristic fragmentation of different isoflavones were unequivocally identified and differentiated. The occurrence of aldehydes such as pentanal, ethanal and methanal was shown to be specifically linked with isoflavone aglycones, daidzein, genistein and glycitein, respectively. Main glycosides such as genistin, daidzin and glycitin as well as the acetyl-, and malonyl forms also showed respective aglycone ions in their spectra fragmentation. Thus positive ion fragmentation was important in the unequivocal confirmation of isoflavones and for revealing the occurrence of other related compounds such as aldehydes in the soymilk.

Comparison of endogenous β -glucosidases and β -galactosidases in selected probiotic bacteria as hydrolysing enzymes in the breakdown of the predominant isoflavone glycosides in soymilk into bioactive isoflavone aglycones is critical for an optimised processing of a probiotic functional food. β -glucosidase activity and β -galactosidase activity of probiotic organisms including *L. acidophilus* ATCC 4461, *L. casei* 2607 and *B. animalis* ssp. *lactis* Bb12 in soymilk was evaluated and correlated with the increase in concentration of isoflavone aglycones during fermentation. The concentrations of isoflavone compounds in soymilk were monitored using a Varian model HPLC with an amperometric electrochemical detector. In all microorganisms, β -glucosidase activity was found to be greater than that of β -galactosidase. The aglycone concentration in the soymilk with *L. acidophilus* 4461, *L. casei* 2607 and *B. animalis* ssp. *lactis* Bb12, increased by 5.37, 5.52 and 6.10 fold, respectively after 15 h of fermentation at 37°C. The maximum hydrolytic potential was also observed at 15 h of fermentation for the three micro-organisms coinciding with peak activities of the two enzymes. β -glucosidase activity was found to be more than 15 times higher than that of β -galactosidase activity in the soymilk for each microorganism

during fermentation. It appears β -glucosidase played a greater role in isoflavone hydrolysis. It is important to determine critical parameters such as which hydrolysing enzyme have a greater impact in the development of a probiotic functional food beverage. In this case it is essential to enhance β -glucosidase activity for its greater role in improving the biological activity of soymilk during processing.

Having established that endogenous β -glucosidase plays a greater role in isoflavone biotransformation, it was essential to compare endogenous and exogenous β -glucosidases for their role in isoflavone biotransformation. β -glucosidase activity of probiotic organisms including *Bifidobacterium animalis* ssp. *lactis* Bb12, *Lactobacillus acidophilus* ATCC 4461 and *Lactobacillus casei* 2607 in soymilk was evaluated and found to relate to the increase in concentration of isoflavone aglycones during fermentation. The concentrations of isoflavone compounds in soymilk were monitored using a Varian model HPLC with an Amperometric electrochemical detector. The aglycone composition, also known as aglycone equivalent ratio, has been considered to be important for delivery of health benefits of isoflavones, was also monitored during fermentation of soymilk. Comparison of the hydrolytic effectiveness of both exogenous and endogenous enzyme during 4 h incubation in soymilk was conducted using the Otieno-Shah (O-S) index. Results showed that exogenous enzyme exhibited faster rate of isoflavone glycoside hydrolysis than that by endogenous enzyme. Highest O-S indices were obtained after 4, 3 and 2 h of incubation with enzyme solution having β -glucosidase activity of 0.288 UmL^{-1} , 0.359 UmL^{-1} , and 0.575 UmL^{-1} resulting into aglycone concentration increments of 5.87, 6.07 and 5.94 fold, respectively. Conversely, aglycone concentration in the soymilk with *B. animalis* ssp. *lactis* Bb12, *L. casei* 2607 and *L. acidophilus* 4461 increased by 3.43, 2.72 and 3.03 fold, respectively after 4 h of fermentation at 37°C . Also, the O-S index of endogenous enzyme was much lower than that of the exogenous enzyme over the same 4 h incubation period. Optimum aglycone equivalent ratios coincided with highest O-S indices and highest aglycone

concentrations in soymilk hydrolysed with exogenous enzyme. The same correlation of O-S indices and highest aglycone concentrations occurred for endogenous enzyme during the 24 h of fermentation. Therefore, obtaining highest aglycone concentration as well as optimum aglycone equivalent ratio could provide a critical beginning point in clinical trials for maximum realisation of the unique health benefits of soy isoflavones. Screening for β -glucosidase activities of probiotic bacteria in soymilk as well as comparing their hydrolytic potentials with that exogenous β -glucosidase could find wide applications in the development of different aglycone rich functional soy beverages.

Professor Nagendra P. Shah *M.Sc., Ph.D*
School of Molecular Sciences
Victoria University
Werribee Campus, Victoria University,
Australia.

CERTIFICATE

This is to certify that this thesis entitled “STABILITY OF BIOACTIVE ISOFLAVONES AND GLYCOLYTIC ENZYMES PRODUCED BY PROBIOTIC BACTERIA IN SOY BASED FOOD DURING PROCESSING AND STORAGE” submitted by Daniel Obed Otieno in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Food Science and Technology at Victoria University is a record of genuine research work conducted by him from September 2003 to January 2007 under my personal guidance and supervision. Furthermore, this thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

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

Date:.....

Acknowledgements

To my supervisor, Prof. Nagendra Shah, the one who has been most influential to my intellectual pursuit in the execution of this PhD program, I am greatly indebted. The moulding received as a result of his professional, thorough, expedient and focused tutelage has resulted into what I consider to be a significant contribution in the realm of scientific knowledge through publications in refereed journals of international repute. For this, I confer a momentous thank you.

I also wish to acknowledge the special role of my associate supervisor Dr. John F. Ashton, Sanitarium Health Food Co., Cooranbong, NSW, Australia, for being a constant source of encouragement, guidance and support through visits, communications and provision of some of the materials critical to the study. It all made this journey worthwhile.

To my colleagues such as Osaana, Lydia, Thuy, Annelise, Lata, Ekta and Wun, I thank for their friendship, support and the helpful camaraderie.

I also wish to acknowledge the tremendous support of the laboratory staff for their assistance. Special mention goes to Dale, the lab manager, Stacey, Ira and Mary Anne. I would also like to thank Joe for the technical assistance and induction with the use of various analytical instruments in the laboratory.

I am immensely grateful to the Australian Research Council for providing an Australian Postgraduate award (Industry) for this research program and I also appreciate the extensive contribution offered by Sanitarium Health Food Company as a co-sponsor of the above scholarship. I am grateful to the thoughtful efforts of Professor Shah and Dr. Ashton for formulating this project and giving me the unique opportunity to undertake the research.

Last but not the least, I am grateful to the creator God for His enabling grace and for fixing in my life such a momentous destiny including a PhD program at Victoria University, Australia. I am unaware of what tomorrow might bring, but I rest my case, my life in His enabling, safe and secure Hands. Whate'er betide, still tis the Lords Hands that leadeth me.

Daniel Obed Otieno

School of Molecular Sciences

Faculty of Science, Engineering and Technology

Victoria University, Werribee Campus, Australia

List of Publications, Conference Presentations and Awards

REFEREED RESEARCH PAPERS

1. Otieno, D.O., Ashton, J.F., Shah, N.P., 2005. Stability of β -glucosidase activity produced by *Bifidobacterium* and *Lactobacillus* spp. in fermented soymilk during processing and storage. *Journal of Food Science*. 70, 4, 236 – 241.
2. Otieno, D.O., Ashton, J.F., Shah, N.P. 2006. Evaluation of enzymic potential for biotransformation of isoflavone phytoestrogen in soymilk by *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus casei*. *Food Research International*. 39, 394 – 407.
3. Otieno, D.O., Ashton, J.F., Shah, N.P., 2006. Stability of isoflavone phytoestrogens in fermented soymilk with *Bifidobacterium animalis* Bb12 during storage at different temperatures. *International Journal of Food Science Technology*. 41, 1182 – 1191.
4. Otieno, D.O., Ashton, J.F., Shah, N.P. 2007a. 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 .
5. Otieno, D.O., Ashton, J.F., Shah, N.P. 2007b. Role of microbial strain and storage temperatures in the degradation of isoflavone phytoestrogens in fermented soymilk with selected β -glucosidase producing *L. casei* strains. *Food Research International*. 40, 3, 371 – 380.
6. Otieno, D. O., Rose, H., Shah, N. P. 2007. Profiling of isoflavones in soymilk from soy protein isolate using extracted ion chromatography and positive ion fragmentation techniques. *Food Chemistry*. 2007. (In Press).

7. Otieno, D. O., Shah, N. P. 2007. A comparison of changes in transformation of isoflavones in soymilk using varying concentrations of exogenous and probiotic-derived endogenous β -glucosidases. *Journal of Applied Microbiology*. 103, 601 - 612.
8. Otieno, D. O., Shah, N. P. 2007. Endogenous β -glucosidase and β -galactosidase activities from selected probiotic microorganisms and their hydrolytic role in isoflavone biotransformation in soymilk. *Journal of Applied Microbiology* (In Press).

CONFERENCE PRESENTATIONS

1. Otieno, D. O., Ashton, J. F., & Shah, N. P. 2004. Stability of β -glucosidase activity produced by *Bifidobacterium* and *Lactobacillus* spp. in fermented soymilk during processing and storage. Poster presentation. 37th Annual AIFST Convention and Exhibition, 25 – 28 July, 2004, Brisbane, Australia.
2. Otieno, D. O., Ashton, J. F., & Shah, N. P. 2006. Exogenous and endogenous β -glucosidases - A comparison of real-time kinetics of isoflavone biotransformation in soymilk. Poster Presentation. IFT Annual Food Expo, 24th – 28th June, 2006, Orlando, Florida.
3. Otieno, D. O., Ashton, J. F., & Shah, N. P. 2006. The effects of microbial strain and storage temperatures in the degradation of bioactive isoflavone phytoestrogens in soymilk fermented with β -glucosidase producing micro-organisms. Poster Presentation. 13th World Congress of Food Science and Technology, 17th – 21st September, 2006, Nantes, France.
4. Otieno, D. O., Ashton, J. F., & Shah, N. P. 2006. Profiling, identification and biotransformation of isoflavones in fermented soymilk using endogenous and exogenous β -glucosidases. Oral Presentation. 13th World Congress of Food Science and Technology, 17th – 21st September, 2006, Nantes, France.

5. Otieno, D. O & Shah, N. P. 2007. Profiling, biotransformation, stability and health potential of isoflavones in soymilk. Oral Presentation. 40th Annual AIFST Convention and Exhibition, 23 – 26 June, 2007, Melbourne, Australia.

AWARDS

1. **Outstanding final year postgraduate research student** in the Faculty of Health, Engineering and Science, **July 2007** in Victoria University. Award of Certificate in recognition of Excellence in Academic Performance and Cash at a faculty dinner party on the 26th July 2007.
2. **Secomb Conference and Travel Fund Scholarship** in 2006 to attend International Scientific Conferences. Certificate of Award given at a faculty dinner party on the 26th July 2007.

Table of Contents

Chapter.....	Page
I. Abstract.....	i
II. Certificate.....	vii
III. Acknowledgements.....	viii
IV. List of Publications, Conferences and Awards.....	x
V. Table of Contents.....	xiii
VI. List of Tables.....	xxiii
VII. List of Figures.....	xxvi
VIII. List of Abbreviations.....	xxx
1.0 General Introduction.....	1
2.0 Review of Literature.....	6
2.1 The history of soy foods.....	6
2.1.1 Growth and Development in the Western World.....	6
2.1.2 Soybeans production around the world.....	7
2.1.3 Utilization of soybeans.....	7
2.2 Overview of soy foods in the market.....	8
2.2.1 Soymilk.....	8
2.2.2 Soymilk yogurt.....	8
2.3 Drivers of market growth of soy foods.....	9
2.3.1 Technological innovations and “Westernisation” of soyfoods.....	9
2.3.2 Incorporation of soyfoods into the product ranges of major food companies.....	9
2.3.3 Consumer awareness of possible soy-health links.....	10

2.4	Phytoestrogens.....	11
2.4.1	Soy isoflavones.....	12
2.4.2	Occurance of soy isoflavones.....	14
2.4.3	Metabolism of soy isoflavone.....	15
2.4.4	Summary of absorption and metabolic fate of isoflavones.....	18
2.5	Health benefits of soy.....	18
2.5.1	Cardiovascular disease.....	18
2.5.2	Cancer and the risk of cancer.....	21
2.5.3	Post-menopausal symptoms.....	25
2.5.3.1	Hot flushes	25
2.5.3.2	Osteoporosis.....	26
2.5.3.3	Estrogen replacement therapy.....	27
2.5.3.4	Cardiovascular function.....	28
2.5.4	Cognitive function and neurologic diseases.....	29
2.5.5	Bone development.....	30
2.6	Profiling of isoflavones in commercial soy bean foods.....	31
2.6.1	Isoflavone content of soy food products in Australian market.....	32
2.7	The stability of soy isoflavones during storage and processing.....	33
2.7.1	Processing and storage-induced changes in soy isoflavones.....	34
2.7.2	Degradation kinetics of soy isoflavones.....	36
2.7.3	Effects of temperature on degradation of isoflavones.....	38
2.8	Probiotic Bacteria.....	40
2.8.1	Genus <i>Lactobacillus</i>	41
2.8.1.1	<i>Lactobacillus acidophilus</i>	42
2.8.1.2	Health benefits of <i>Lactobacillus acidophilus</i>	43

2.8.1.3	<i>Lactobacillus acidophilus</i> and Food Production.....	44
2.8.2	<i>Lactobacillus casei</i>	44
2.8.3	Genus <i>Bifidobacterium</i>	44
2.8.4	Metabolic activity of probiotic bacteria in soymilk.....	47
2.9	Challenges with soy isoflavone-based nutritional supplements	48
3.0 Stability of β-Glucosidase Activity Produced by <i>Bifidobacterium</i> and <i>Lactobacillus</i> spp. in Fermented Soymilk During Processing and Storage.....50		
3.1	INTRODUCTION.....	50
3.2	MATERIALS AND METHODS.....	51
3.2.1	Bacteria.....	51
3.2.2	Soymilk manufacture.....	52
3.2.3	Fermentation and storage.....	52
3.2.4	Assay for β -glucosidase activity in Soymilk.....	52
3.2.5	Experimental design.....	53
3.2.6	Statistical analysis.....	54
3.3	RESULTS AND DISCUSSION.....	54
3.3.1	β -Glucosidase activity the microorganisms.....	54
3.3.2	Stability of β -glucosidase during storage at different temperatures.....	56
3.3.3	Stability of β -glucosidase at different storage temperatures; - a critical analysis for 5 weeks.....	58
3.4	CONCLUSIONS.....	60

4.0	Evaluation of Enzymic Potential for Biotransformation of Isoflavone Phytoestrogen in Soymilk by <i>Bifidobacterium animalis</i>, <i>Lactobacillus acidophilus</i> and <i>Lactobacillus casei</i>.....	76
4.1	INTRODUCTION.....	76
4.2	MATERIALS AND METHODS.....	78
4.2.1	Bacteria.....	78
4.2.2	Bacterial growth media.....	78
4.2.3	Soymilk manufacture.....	78
4.2.4	Assay for β -glucosidase activity in soymilk.....	78
4.2.5	Fermentation of soymilk with probiotics.....	79
4.2.6	Extraction of isoflavones for HPLC analysis.....	79
4.2.7	Isoflavone standards.....	80
4.2.8	Reversed-phase HPLC apparatus and reagents.....	80
4.2.9	HPLC analysis of isoflavones.....	81
4.2.10	Statistical analysis.....	81
4.3	RESULTS AND DISCUSSION.....	81
4.3.1	β -Glucosidase activity in soymilk.....	81
4.3.2	HPLC analysis of isoflavones in soymilk.....	82
4.3.3	Biotransformation of isoflavone compounds in fermented soymilk.....	84
4.3.4	Evaluation of the hydrolytic potential of probiotics in the isoflavone biotransformation process.....	88
4.4	CONCLUSIONS.....	89

5.0	Stability of Isoflavone Phytoestrogens in Fermented Soymilk with <i>Bifidobacterium animalis</i> ssp. <i>lactis</i> Bb12 During Storage at Different Temperatures.....	105
5.1	INTRODUCTION.....	105
5.2	MATERIALS AND METHODS.....	106
5.2.1	Bacteria.....	106
5.2.2	Bacterial growth media.....	107
5.2.3	Soymilk manufacture.....	107
5.2.4	Fermentation of soymilk with <i>B. animalis</i> Bb12.....	107
5.2.5	Extraction of isoflavones for HPLC analysis.....	107
5.2.6	Reversed-phase HPLC apparatus and reagents.....	108
5.2.7	HPLC analysis of isoflavones.....	109
5.2.8	Statistical analysis.....	109
5.3	RESULTS AND DISCUSSION.....	110
5.3.1	Changes in the concentration of isoflavones during storage at different temperatures.....	110
5.3.2	Kinetics of soy isoflavone degradation during storage.....	114
5.4	CONCLUSIONS.....	118
6.0	Isoflavone Phytoestrogen Degradation in Fermented Soymilk in with Selected β-Glucosidase Producing <i>L. acidophilus</i> strains During Storage at Different Temperatures.....	128
6.1	INTRODUCTION.....	128
6.2	MATERIALS AND METHODS.....	129
6.2.1	Microorganisms.....	129

6.2.2	Bacterial growth media.....	129
6.2.3	Soymilk manufacture.....	130
6.2.4	Fermentation of soymilk with <i>L. acidophilus</i> 4461, <i>L. acidophilus</i> ATCC 4962 and <i>L. acidophilus</i> ATCC 33200.....	130
6.2.5	Extraction of isoflavones from freeze dried samples.....	130
6.2.6	Reversed-phase HPLC apparatus and reagents.....	131
6.2.7	HPLC analysis of isoflavones.....	132
6.2.8	Isoflavonoid identification.....	132
6.2.9	Standard solutions and calibration curves.....	132
6.2.10	Statistical analysis of data.....	133
6.2.11	First order kinetics of isoflavone degradation.....	133
6.3	RESULTS AND DISCUSSION.....	134
6.3.1	Changes in the concentration of isoflavones in fermented soymilk during storage.....	134
6.3.2	Degradation rates of isoflavone glucosides and aglycones as influenced by storage temperature.....	137
6.3.3	Degradation rates of isoflavone compounds in soymilk as influenced by <i>L. acidophilus</i> strain and molecular structure.....	139
6.3.4	Mechanisms of isoflavone degradation in fermented soymilk at different storage temperatures.....	141
6.4	CONCLUSIONS.....	143
7.0	Role of Microbial Strain and Storage Temperatures in the Degradation of Isoflavone Phytoestrogens in Fermented Soymilk with Selected β-Glucosidase Producing <i>L. casei</i> strains.....	151

7.1	INTRODUCTION.....	151
7.2	MATERIALS AND METHODS.....	153
7.2.1	Bacteria.....	153
7.2.2	Soymilk manufacture.....	153
7.2.3	Fermentation of soymilk with <i>L. casei</i> 2607 and <i>L. casei</i> ASCC 290.....	153
7.2.4	Extraction of isoflavones from freeze dried samples.....	154
7.2.5	Reversed-phase HPLC apparatus and reagents.....	154
7.2.6	HPLC analysis of isoflavones.....	155
7.2.7	Isoflavone identification.....	155
7.2.8	Standard solutions and calibration curves.....	155
7.2.9	Statistical analysis of data.....	156
7.3	RESULTS AND DISCUSSION.....	156
7.3.1	Isoflavone concentrations at peak β -glucosidase activity.....	156
7.3.2	Concentration of isoflavones in fermented soymilk during storage.....	157
7.3.3	The kinetics of soy isoflavone degradation in fermented soymilk during storage.....	159
7.3.4	Degradation rates of isoflavones in fermented soymilk as influenced by storage temperature and molecular structure.....	161
7.3.5	Overall effects of storage temperature on the stability of isoflavones in fermented soymilk.....	163
7.3.6	Comparison of <i>L. casei</i> ASCC 290 and <i>L. casei</i> 2607 and their role in isoflavone degradation in soymilk during storage.....	164
7.4	CONCLUSIONS.....	165

8.0	Profiling of Isoflavones in Soymilk from Soy Protein Isolate using Extracted Ion Chromatography and Positive Ion Fragmentation Techniques.....	175
8.1	INTRODUCTION.....	175
8.2	MATERIALS AND METHODS.....	177
8.2.1	Bacteria.....	177
8.2.2	Bacterial growth media.....	177
8.2.3	Soymilk manufacture.....	178
8.2.4	Fermentation of soymilk with <i>B. animalis</i> ssp. <i>lactis</i> Bb12.....	178
8.2.5	Extraction of isoflavones for LC-MS/MS analysis.....	178
8.2.6	Isoflavone standards.....	179
8.2.7	LC-MS and LC-MS/MS instrumentation.....	179
8.2.8	Statistical analysis.....	180
8.3	RESULTS AND DISCUSSION.....	180
8.3.1	Compositional variations of isoflavones during fermentation.....	180
8.3.2	Identification and quantitation of isoflavone compounds.....	181
8.3.3	Separation of existing isoflavone compounds in fermented and unfermented soymilk.....	183
8.3.4	Product ion analysis of isoflavone glucosides in soymilk.....	184
8.3.5	Comparison of product ions obtained in ESI-MS/MS of the malonyl-, and acetylglucosides detected in soymilk.....	186
8.4	CONCLUSIONS.....	187

9.0	Endogenous β-Glucosidase and β-Galactosidase Activities from Selected Probiotic Microorganisms and their Hydrolytic Role in Isoflavone Biotransformation in Soymilk.....	199
9.1	INTRODUCTION.....	199
9.2	MATERIALS AND METHODS.....	201
9.2.1	Bacterial strains.....	201
9.2.2	Soymilk manufacture.....	201
9.2.3	Assay for β -glucosidase and β -galactosidase enzymes from <i>L. acidophilus</i> ATCC 4461, <i>L. casei</i> 2607 and <i>B. animalis</i> ssp. <i>lactis</i> Bb12 and in soymilk.....	201
9.2.4	Quantification of β -glucosidase activity in soymilk.....	202
9.2.5	Quantification of β -galactosidase activity in soymilk.....	202
9.2.6	Extraction of isoflavones from freeze dried samples.....	203
9.2.7	HPLC analysis of isoflavones.....	203
9.2.8	Otieno – Shah (O – S) index.....	204
9.2.9	Statistical analysis of data.....	204
9.3	RESULTS.....	205
9.3.1	β -Glucosidase activity of the probiotic microorganisms in soymilk.....	205
9.3.2	β -Galactosidase activity of probiotic microorganisms in fermented soymilk.....	205
9.3.3	Isoflavone aglycone yields from glucoside hydrolysis.....	205
9.3.4	Comparative increase of isoflavone aglycones from isoflavone glycosides in soymilk during hydrolysis by microbial enzymes.....	206
9.3.5	Evaluation of the hydrolytic potential of the endogenous enzymes in isoflavone transformation in soymilk	

	during 24 h fermentation.....	206
9.4	DISCUSSION.....	206
9.5	CONCLUSIONS.....	210
10.0	A Comparison of Changes in the Transformation of Isoflavones in Soy milk using Varying Concentrations of Exogenous and Probiotic-Derived Endogenous β-Glucosidases.....	216
10.1	INTRODUCTION.....	216
10.2	MATERIALS AND METHODS.....	218
	10.2.1 Bacterial strains.....	218
	10.2.2 Soy milk manufacture.....	218
	10.2.3 Preparation of enzyme solutions and assay in soy milk.....	219
	10.2.4 Fermentation of soy milk with <i>B. animalis</i> ssp. <i>lactis</i> Bb12, <i>L. casei</i> 2607 and <i>L. acidophilus</i> ATCC 4461 and with exogenous β -glucosidase.....	219
	10.2.5 Extraction of isoflavones from freeze dried samples.....	219
	10.2.6 Isoflavone standards.....	220
	10.2.7 HPLC analysis of isoflavones.....	220
	10.2.8 Otieno – Shah (O – S) index.....	221
	10.2.9 Statistical analysis of data.....	221
10.3	RESULTS.....	221
	10.3.1 Changes in isoflavone concentration in soy milk using exogenous β -glucosidase.....	221
	10.3.2 Aglycone equivalent ratios for isoflavone aglycones in soy milk.....	222
	10.3.3 Comparison of the effects of exogenous and endogenous	

β-glucosidase in increasing the aglycone concentration in the soymilk during the 4 h incubation.....	222
10.3.4 Evaluation of the hydrolytic potential of exogenous and endogenous enzymes in isoflavone transformation in soymilk.....	223
10.4 DISCUSSION.....	223
10.5 CONCLUSIONS.....	229
11.0 Overall Conclusions and Future Research Direction.....	241
12.0 List of References.....	246
Appendices.....	276
Appendix A. Method for enzyme assay for β-glucosidase in Soymilk using p-nitrophenol as Substrate.....	276
Appendix B. Method for Enzyme assay for β-galactosidase in Soymilk using o-nitrophenol as Substrate.....	278
Appendix C. Quantification of isoflavone compounds found in fermented and non-fermented soymilk samples using HPLC with an internal standard (ISTD).....	280
Appendix D. Method for calculating the concentration of malonyl- and acetyl-glycoside isoflavone compounds in soymilk analysed with HPLC.....	281
Appendix E. Published Chapters as appearing in various peer reviewed scientific Journals of repute.....	285

List of Tables

Table.....	Page
2.1 World Soybean Production by Major Producers.....	7
2.2 Isoflavone levels in some of the foods in an Australian Supermarket.....	32
2.3 Kinetic parameters for degradation of soy isoflavones in different products at different temperatures	39
2.4 Arrangement of the Genus <i>Lactobacillus</i>	42
3.1 Peak enzyme activity in fermented soymilk at 24 h of incubation at 37°C.....	61
3.2 β -Glucosidase activity ¹ of the selected probiotic micro-organisms in soymilk incubated for 12, 24, 36, and 48 h.....	62
3.3 β -Glucosidase activity ¹ of <i>Lactobacillus acidophilus</i> 33200 in soymilk during storage at different temperatures.....	63
3.4 β -Glucosidase activity ¹ of <i>Bifidobacterium animalis</i> BB12 in soymilk during storage at different temperatures.....	64
3.5 β -Glucosidase activity ¹ of <i>Lactobacillus casei</i> 2607 in soymilk during storage at different temperatures.....	65
3.6 β -Glucosidase activity ¹ of <i>Lactobacillus acidophilus</i> 4962 in soymilk during storage at different temperatures.....	66
3.7 β -glucosidase activity ¹ of <i>Lactobacillus acidophilus</i> 4461 in soymilk during storage at different temperatures.....	67
3.8 β -glucosidase activity ¹ of <i>Lactobacillus casei</i> ASCC 290 in soymilk during storage at different temperatures.....	68
4.1 Concentration of isoflavone isomers (mg/ 100 mL) in soymilk fermented by <i>L. acidophilus</i> 33200 for 12, 24, 36 and 48 h of incubation at 37°C.....	91
4.2 Concentration of isoflavone isomers (mg/ 100 mL) in soymilk fermented by <i>B. animalis</i> BB12 for 12, 24, 36 and 48 h of incubation at 37°C.....	92
4.3 Concentration of isoflavone isomers (mg/ 100 mL) in soymilk fermented by <i>L. casei</i> 2607 for 12, 24, 36 and 48 h of incubation at 37°C.....	93
4.4 Concentration of isoflavone isomers (mg/ 100 mL) in soymilk	

	fermented by <i>L. acidophilus</i> 4962 for 12, 24, 36 and 48 h of incubation at 37°C.....	94
4.5	Concentration of isoflavone isomers (mg/ 100 mL) in soymilk fermented by <i>L. acidophilus</i> 4461 for 12, 24, 36 and 48 h of incubation at 37°C.....	95
4.6	Concentration of isoflavone isomers (mg/ 100 mL) in soymilk fermented by <i>L. casei</i> ASCC 290 for 12, 24, 36 and 48 h of incubation at 37°.....	96
4.7	Otieno – Shah (O –S) index (Isoflavone hydrolytic index) of 6 micro organisms in soymilk at peak β -glucosidase activity during incubation at 37°C.....	97
5.1	Concentration of isoflavones (mg/ 100 mL) in fermented soymilk using <i>B. animalis</i> BB12 during storage at –80°C.....	119
5.2	Concentration of isoflavones (mg/100 mL) in fermented soymilk using <i>B. animalis</i> Bb12 during storage at 4°C.....	120
5.3	Concentration of isoflavones (mg/100 mL) in fermented soymilk using <i>B. animalis</i> Bb12 during storage at 25°C.....	121
5.4	Concentration of isoflavones (mg/100 mL) in fermented soymilk using <i>B. animalis</i> BB12 during storage at 37°C.....	122
5.5	Kinetic parameters for degradation of soy isoflavones in fermented soymilk using <i>B. animalis</i> Bb12 during storage.....	123
6.1	Concentration of isoflavones (ng/ μ L) in fermented soymilk using 3 <i>L. acidophilus</i> strains during storage at different temperatures.....	144
6.2	First order kinetic parameters for degradation of soy isoflavones in fermented soymilk using 3 strains of <i>Lactobacillus acidophilus</i> during storage at -80°C, 4°C, 25°C and 37°C.....	145
7.1	Concentration of isoflavones (ng/ μ L) in fermented soymilk using two <i>L. casei</i> strains during storage at different temperatures.....	167
7.2	First order kinetic parameters for degradation of soy isoflavones in fermented soymilk using <i>Lactobacillus casei</i> ASCC 290 and <i>Lactobacillus casei</i> 2607 during storage at -80°C, 4°C, 24.8°C and 37°C.....	168
8.1	Concentration of isoflavones (μ g/mL) in fermented soymilk using <i>Bifidobacterium animalis</i> ssp. <i>lactis</i> Bb12.....	188

8.2	Summary of product ion data for protonated isoflavones in soymilk during fragmentation using ESI-LC MS/MS.....	189
8.3	Summary of the <i>m/z</i> , peaks of fragments and the molecular weights of isoflavones in the soymilk using ESI-MS/MS on positive fragmentation.....	190
9.1	β -Glucosidase activity (U mL^{-1}) of selected probiotics in fermented soymilk at 37°C for 24 h.....	211
9.2	β -Galactosidase activity (U mL^{-1}) of selected probiotics in fermented soymilk at 37°C for 24 h.....	212
9.3	Increments of total aglycone concentration in soymilk during 24 h incubation with selected probiotic micro-organisms.....	213
10.1	Aglycone equivalent ratios for isoflavone aglycones in soymilk during Incubation/Fermentation period at 37°C with different concentrations of exogenous β -glucosidase and microorganisms.....	231
10.2	Increments of total aglycone concentration in soymilk during 4h incubation with different concentrations of exogenous β -glucosidase and micro-organisms.....	232

List of Figures

Figure.....	Page
2.1 Chemical structures of the human estrogen, 17 β -estradiol, and the classes of phytoestrogens compounds, isoflavones, lignans, coumestans, and resorcylic acid lactones.....	11
2.2 Chemical structure of mammalian lignan, enterodiol (1) and the metabolised Intestinal lignan, enterolactone (2).....	12
2.3 Four chemical forms of three analogues of isoflavones found in soybeans.....	13
2.4 Chemical structures of the isoflavones, daidzein, genistein and glycitein, in aglycone form.....	13
2.5 Chemical structure of a soy isoflavone glucoside.....	14
2.6 The metabolism of daidzein.....	16
2.7 The metabolism of genistein.....	17
2.8 Genistin degradation in soymilk stored at 90°C.....	37
2.9 Schematic diagram of bifidobacterial sugar metabolism.....	45
3.1 β -Glucosidase activity in soymilk by <i>L. acidophilus</i> 33200 during storage at different temperatures.....	69
3.2 β -Glucosidase activity in soymilk by <i>B. animalis</i> Bb12 during storage at different temperatures.....	70
3.3 β -Glucosidase activity in soymilk by <i>L. casei</i> 2607 during storage at different temperatures.....	71
3.4 β -Glucosidase activity in soymilk by <i>L. acidophilus</i> 4962 during storage at different temperatures.....	72
3.5 β -Glucosidase activity in soymilk by <i>L. acidophilus</i> 4461 during storage at different temperatures.....	73
3.6 β -Glucosidase activity in soymilk by <i>L. casei</i> ASCC 290 during storage at different temperatures.....	74
3.7 pH changes in soymilk during fermentation with each of the 6 microorganisms at 37°C.....	75
4.1. Reversed-phase HPLC chromatogram showing approximate retention times of isoflavone isomer metabolites (at wavelenth of 260 nm) found in unfermented soymilk.....	98
4.2 Percentage change in isoflavone composition during fermentation using <i>L. acidophilus</i> 33200 at 37°C.....	99
4.3 Percentage change in isoflavone composition during fermentation	

	with <i>B. animalis</i> BB12 at 37°C.....	100
4.4	Percentage change in isoflavone composition during fermentation using <i>L. casei</i> 2607 at 37°C.....	101
4.5	Percentage change in isoflavone composition during fermentation using <i>L. acidophilus</i> 4962 at 37°C.....	102
4.6	Percentage change in isoflavone composition during fermentation using <i>L. acidophilus</i> 4461 at 37°C.....	103
4.7	Percentage change in isoflavone composition during fermentation with <i>L. casei</i> ASCC 290 at 37°C.....	104
5.1	Isoflavone degradation in fermented soymilk during storage at -80°C.....	124
5.2	Isoflavone degradation in fermented soymilk during storage at 4°C.....	125
5.3	Isoflavone degradation in fermented soymilk during storage at 25°C.....	126
5.4	Isoflavone degradation in fermented soymilk during storage at 37°C.....	127
6.1	Isoflavone degradation in fermented soymilk using <i>L. acidophilus</i> ATCC 4962 during storage at -80°C.....	146
6.2	Isoflavone degradation in fermented soymilk using <i>L. acidophilus</i> 4461 during storage at 4°C.....	147
6.3	Isoflavone degradation in fermented soymilk using <i>L. acidophilus</i> ATCC 33200 during storage at 25°C.....	148
6.4	Isoflavone degradation in fermented soymilk using <i>L. acidophilus</i> ATCC 4461 during storage at 37°C.....	149
6.5	Effect of storage temperature on the degradation of isoflavones in fermented soymilk.....	150
7.1	Isoflavone degradation in fermented soymilk using <i>L. casei</i> ASCC 290 during storage at - 80°C.....	169
7.2	Isoflavone degradation in fermented soymilk using <i>L. casei</i> 2607 during storage at 4°C.....	170
7.3	Isoflavone degradation in fermented soymilk using <i>L. casei</i> ASCC 290 during storage at 25°C.....	171
7.4	Isoflavone degradation in fermented soymilk using <i>L. casei</i> 2607 during storage at 37°C.....	172
7.5	Effect of storage temperature on the degradation of isoflavones in fermented soymilk.....	173
7.6	Comparison of the influence of the two <i>L. casei</i> strains on the degradation	

	of isoflavones in fermented soymilk during storage at different temperatures.....	174
8.1	Structures of isoflavone aglycones daidzein, genistein and glycitein showing the B ring attachment at carbon 3.....	191
8.2	Electro-spray ionisation chromatogram using LC-MS of isoflavones in unfermented soymilk (0 h) made from soy protein isolate (SPI; SUPRO 590).....	192
8.3	Electro-spray ionisation chromatogram of isoflavones using LC-MS in soymilk made from SPI; SUPRO 590, fermented with <i>B. animalis</i> ssp. <i>lactis</i> Bb12 after 12h at 37°C.....	193
8.4	Separation and quantitation of malonylgenistin (bigger peak-EIC 519) and acetylglycitin (smaller peak-EIC 489) in fermented soymilk using LC-MS.....	194
8.4	Products ions obtained in ESI-MS/MS experiments of protonated isoflavone glycosides (M + H) daidzin, genistin and glycitin (A, B, and C), respectively in soymilk.....	195
8.5	Comparison of the products ions obtained in ESI-MS/MS experiments of protonated standards of puerarin (A; C-glycosylated) and daidzin (B; O-glycosylated), respectively.....	196
8.6	Products ions obtained in ESI-MS/MS experiments of protonated isoflavone aglycones daidzein, genistein and glycitein (D, E, and F) in soymilk, respectively.....	197
8.7	Products ions obtained in ESI-MS/MS experiments of protonated isoflavones of malonyl-, and acetylglucosides (G, H, I, J, and K) product ions, respectively in soymilk.....	198
9.1	Total aglycone increment and total glycoside decrement values in fermented soymilk with selected probiotic microorganisms.....	214
9.2	Hydrolytic indices (Otieno Shah (O-S) indices) of isoflavone hydrolysis in soymilk using probiotic micro-organisms during 24 h fermentation at 37°C.....	215
10.1	Changes in isoflavone concentration in soymilk during incubation at 37°C using 0.288 UmL ⁻¹ of exogenous β-glucosidase.....	233
10.2	Changes in isoflavone concentration in soymilk during incubation at 37°C using 0.359 UmL ⁻¹ of exogenous β-glucosidase.....	234
10.3	Changes in isoflavone concentration in soymilk during incubation at 37°C	

	using 0.575 U mL^{-1} of exogenous β -glucosidase.....	235
10.4	Comparison of hydrolytic potential (Otieno Shah (O-S) index) of probiotic micro-organisms and exogenous β -glucosidase in isoflavone transformation in the soymilk during 4 h fermentation/incubation at 37°C	236
10.5	Hydrolytic indices (Otieno Shah (O-S) indices) of isoflavone hydrolysis in soymilk using probiotic micro-organisms during 24 h fermentation at 37°C	237
10.6	Changes in isoflavone concentration ($\mu\text{g/mL}$) in soymilk during fermentation with <i>Bifidobacterium animalis</i> ssp. <i>lactis</i> Bb12.....	238
10.7	Changes in isoflavone concentration ($\mu\text{g/mL}$) in soymilk during fermentation with <i>Lactobacillus acidophilus</i> ATCC4461.....	239
10.8	Changes in isoflavone concentration ($\mu\text{g/mL}$) in soymilk during fermentation with <i>Lactobacillus casei</i> 2607.....	240

List of Abbreviations

ADME – Absorption, distribution, metabolism and excretion

AHA - American Heart Association

AIQ - Aglycone increment quotient

ANOVA - One-way analysis of variance

ASCC - Australian Starter Culture Centre

ATCC - American Type Culture Centre

Bb12- *Bifidobacterium animalis* ssp. *lactis* Bb12

BL - *Bifidobacterium longum*

C_t - Concentration of isoflavone at time t

C_o - Initial isoflavone concentration

CE - Capillary electrophoresis

CHD - Coronary heart disease

CO₂ - Carbon dioxide

C_p - Heat capacities

CVD - Cardiovascular disease

DSC - Differential scanning calorimetry

E_a - Activation energy

EC - Epicatechin

EGC – Epigallocatechin

EGCG - Epigallocatechin gallate

EIC – Electrospray ionization chromatogram

ESI-MS - Electrospray ionization mass spectrometry

F6PPK - Fructose-6-phosphate phosphoketolase

FAO - Food and Agriculture Organisation of the United Nations

FDA - Food and Drug Administration, USA

FMD - Flow-mediated vasodilation

GRQ - Glycoside reduction quotient

H - Enthalpies

HDL - High density lipoprotein cholesterol

HPLC - High performance liquid chromatography

HRT - Estrogen replacement therapy

ISTD - Internal standard

LA - Lactobacillus acidophilus

LC - Lactobacillus casei

LC-MS – Liquid chromatography mass spectrometry

LDL- Low density lipoprotein cholesterol

MRS – De mann Rogosa and Sharpe broth

MS - Mass spectrometry

MW – Molecular weight

NAD(P)H –

ND – Not Detected

ODMA - *O*-desmethylangolensin

ONPG - *O*-nitrophenyl β -D-galactopyranoside

O-S - Otieno – Shah index

PAR – Peak area response

ρ NPG- ρ -nitrophenyl β -D-glucofuranoside

SPI SUPRO[®] 590 – Soy Protein Isolate SUPRO 590.

UV - Ultraviolet

WHO - World Health Organisation

$\mu\text{g/mL}$ - microgram per millilitre

mg/mL – milligram per millilitre

mL - millilitre

v/v – volume per volume

UmL^{-1} - Units of enzyme per millilitre

1.0 General Introduction

Soyfood utilization around the world varies widely. Asia utilizes soybeans primarily as traditional foods such as tofu, soymilk, and fermented products, whereas Western nations consume soybeans in the form of refined soy protein ingredients used in food processing. The consumption of soy products in Asia is based on longstanding traditional eating habits and food production methods. In Western nations including Australia, it is still regarded as a new phenomenon, although it is gaining increased acceptance (Riaz, 2006). Soybeans in food applications especially became very popular after the Food and Drug Administration approved a health claim for soy protein in 1999. As a result, soy ingredients have had an increased application in diverse food systems hence playing a major role in food functionality.

A very important component of soy ingredients is a group of compounds known as phytoestrogens. They are phenolic compounds found mostly in plants and have structural similarity to human estrogens, therefore possessing a biological activity like that of estrogen (Murkies *et al.*, 1995). There are three main classes of phytoestrogens namely, isoflavones, coumestans and lignans. The soybeans in particular provide the most abundant source of isoflavones (Coward *et al.* 1998; Reinli & Block, 1996) predominantly found in β -glycoside forms which are biologically inactive comprising of 80-95% of the isoflavone concentration (King & Bignell, 2000). On the other hand, the aglycone isoflavones forms are biologically active, possessing estrogenic properties that have been linked to prevention and potential treatment of diseases. These compounds are currently heralded to offer potential alternative therapies for a range of hormone-dependent conditions including cancer, menopausal symptoms, cardiovascular disease, osteoporosis as well as prostate cancer. The aglycone isomers are able to bind to the estrogen receptor sites thereby mimicking the functions of estradiol in the human body (Setchell & Cassidy, 1999). The structural element important in binding to the estrogen receptor sites is the phenolic ring which isoflavones also possess. There is a lot of information

from epidemiological evidence and experimental data in animal studies (Anderson & Garner, 1997; Cassidy, 1996; Knight & Eden, 1996) suggesting beneficial effects of isoflavones on human health.

The isoflavone content of any soyfood including soymilk can be established through analytical procedures involving the use of high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS) techniques. This is important in establishing the isoflavone profile before, during and after fermentation. Although a high proportion of foods contain soy products, mostly as soy oils and soy lecithins, these soy based products are devoid of isoflavones. As a result, the average daily dietary intake of isoflavones in the western populations is typically negligible (<1 mg/d). Asian populations with their high intake (50-70 mg/d) of soy derived isoflavones are known to have the lowest incidence of osteoporosis, menopausal symptoms, cardiovascular disease and cancer (Nagata *et al.* 1998). The chemical form in which isoflavones occurs is an important consideration because it may influence the biological activity, the bioavailability and therefore the physiological effects of these dietary constituents.

Intestinal microflora especially probiotic bacteria play a key role in the metabolism and bioavailability of isoflavones as they hydrolyse the glycoside components using their indigenous β -glucosidase and β -galactosidases in the jejunum, releasing the bioactive aglycone isoflavone form (Setchell, 2000). Aglycone forms have also been found to absorb faster and in higher amounts in human than their respective glycoside forms (Izumi *et al.* 2000). Intestinal bacteria are known to convert daidzein to its metabolite, equol, which is more potent estrogenic compound than its precursor (Setchell & Cassidy, 1999). Thus the use of probiotic bacteria to improve the biological activity of soy based products during processing formed an integral part of this study. *Bifidobacterium*, the predominant member of the intestinal microbiota, is able to preferentially metabolise hexose sugars for growth (Ballongue, 1993). Hence, *Bifidobacterium*,

Lactobacillus acidophilus and *L. casei* were investigated for their potential to hydrolyse isoflavone glycosides while using the sugars as prebiotics for their growth and metabolic activities. Tsangalis *et al.* (2002) established that *B. animalis* (Bb12) is the best producer of β -glucosidase enzymes among *Bifidobacterium* strains. Their maximum enzymatic activity occurred at 24 hrs. On the other hand, *L. acidophilus* and *L. casei* strains have not been studied for their potential to produce β -glucosidase. Therefore, it was important to include these microorganisms in this study in order to ascertain their β -glucosidase activities in soymilk, hence role in biotransformation of isoflavones to improve biological activity in soymilk.

The combined effects of soy (rich source of isoflavones) and probiotics and their effects on disease prevention have not been reported in literature. The importance of the hydrolysing enzymes obtained from these microorganisms could not be fully appreciated until their stability during storage at different temperatures is established. A correlation between β -glucosidase activity and the extent of biotransformation of isoflavone glycosides to aglycone forms was also ascertained in this study in order to optimise the processing and storage of the functional soy based product. There is very little information on the stability of bioactive isoflavones during processing and storage as well as that of probiotic bacteria producing high amounts of β -glucosidase. It is also important to establish means of enhancing the activity of the hydrolysing enzymes during processing and storage in order to guarantee delivery of high levels of bioactive isoflavones and probiotic bacteria to humans for health benefits.

Functional probiotic foods are becoming increasingly popular in the diets of people in Australia and in the western world. A number of health benefits have been claimed for probiotic bacteria such a *L. acidophilus* and *Bifidobacterium* and their inclusion in the diet is important for good health. However, consumption of these micro-organisms in soymilk is an ideal way of re-establishing the balance of intestinal flora after an antibiotic treatment or stress as well as

benefiting from the occurrence of increased concentrations of bioactive isoflavone aglycones. In a recent functional trend survey, manufacturers developing functional foods ranked soy protein as the second key ingredient after isoflavones, for their growing importance in food formulations. This research aimed at establishing optimised conditions for high level of bioactive isoflavones in soymilk during fermentation with probiotic microorganisms, leading to a product with health benefits.

As a consequence of this research, it is expected that the twin beneficial attributes of bioactive aglycones and probiotics of a soy-based food will create a market potential for a range of new health based functional foods. This inevitably will result in great economic significance to the food industry in general and soy industry in particular, thereby contributing to Australia's economic advancement. Therefore, the main aim of this research was to study the stability of bioactive isoflavones aglycones and probiotic bacteria in soy based food, yogurt in particular, during processing and storage. The specific objectives were:

- (a) To ascertain the best conditions for producing high levels of β -glucosidase and β -galactosidase by selected probiotic microorganisms in order to achieve highest bioconversion of isoflavone glycosides to bioactive isoflavone aglycones,
- (b) To assess the stability of β -glucosidase producing ability of selected probiotic microorganisms during processing and storage,
- (c) To assess the stability of bioactive isoflavone constituents during processing and storage of fermented soymilk with selected probiotic microorganisms,
- (d) To carry out a feasibility study of industrial scale manufacture of soy based product (particularly yoghurt) containing high levels of bioactive isoflavones and selected probiotic microorganisms.

There are 12 chapters in this thesis. Chapter 1.0 is the general introduction. Chapter 2.0 contains the review of literature. Chapter 3.0 deals with the study of β -glucosidase activity of some 15 microorganisms in fermented soymilk as well as the stability of the enzyme at different storage temperatures. Chapter 4.0 reports on a special technique on evaluation of the hydrolytic potential of each microorganism in the biotransformation of isoflavones. Chapters 5.0, 6.0 and 7.0 focus on stability of the various forms of isoflavones in soymilk during storage at different temperatures as influenced by microbial enzymes. Chapter 8.0 describes profiling of isoflavones in soy protein isolate using extracted ion chromatography and positive ion fragmentation techniques, while Chapter 9.0 reports on the comparative activities of crude endogenous β -glucosidases and β -galactosidases produced by probiotic microorganisms in order to determine which of the two enzymes plays a greater role in the hydrolysis of isoflavone glycosides. Chapter 10.0 compares hydrolytic efficacy of endogenous and exogenous β -glucosidases on isoflavones. Overall conclusions and future research directions are described in Chapter 11.0, and a list of references is given in Chapter 12.0.

2.0 Literature Review

2.1 The history of soyfoods

Soy is considered a new food for many in the Western society but the Chinese have considered it an important source of nutrition for almost 5000 years. The first reference to soybeans, in a list of Chinese plants, dates back to 2853 B.C., and ancient writings repeatedly refer to it as one of the five sacred grains essential to Chinese civilization (Riaz, 2006). Soybeans and soy foods were officially introduced to the Western society at the beginning of the twentieth century. There was a wide-spread use of soybean throughout the Asian continent in the early part of the last millennium as people in each region developed their own unique soyfoods based on tradition, culture, climate, and local taste preferences. Some soyfoods, such as soy sauce and soymilk, have been accepted by Westerners wholeheartedly, particularly for the past several decades, whereas others will take more time for acceptance (Liu 1997). A whole new class of soy foods have been developed, including soy ice cream, soy yogurt, veggie burgers, soy sausage, and soy flour pancakes and together they have been dubbed “second generation of soy foods”.

2.1.1 Growth and development in the Western world

The European missionaries and traders who travelled to Asia during the 1600s and 1700s noted in their travel journals about traditional soy foods, such as tofu and soymilk which they encountered in the cultures they explored. But it was not until Asians began to emigrate to Europe and North America during the 1800s that soy foods began taking root for consistent use and consumption in the United States. Several Chinese tofu and soymilk shops were established in cities with large Asian populations in Europe and on the East and West coasts of the United States. However, throughout the 19th century, soy foods tended to be made in small, family-run shops and were distributed and consumed primarily in Asian neighbourhoods. During the 1920, a number of smaller companies began making tofu especially in Tennessee and California as a low-cost

source of protein in the production of meat substitutes, and, during both World Wars, large amounts of soy flour were used to help offset meat shortages.

2.1.2 Soybeans production around the world

Eventhough United States is still the largest soybean exporting country in 2006, it has however lost the dominant position once had in the global soy trade. Brazil, Argentina, and India have all become major producers as the world's demand for soy as food, vegetable oil, and animal feed continued to increase. Given the amount of available arable land and water resources in Brazil, along with its low labour costs, it is expected that Brazil will eventually become the leading soybean-producing nation (Golbitz & Jordan, 2006).

Table 2.1 World soybean production by major producers

	Production (million metric tons)				
	2000/01	2001/02	2002/03	2003/04	2004/05
United States	75.06	78.67	75.01	66.78	85.48
Brazil	39.50	43.50	52.00	52.60	63.00
Argentina	27.80	30.00	35.50	34.00	39.00
China	15.40	15.41	16.51	15.40	18.00
India	5.25	5.40	4.00	6.80	6.50
Paraguay	3.50	3.55	4.50	4.00	5.00
All others	9.42	8.60	9.60	10.23	11.64
Total	175.93	185.12	197.12	189.81	227.63

Source: Department of Agriculture estimates, March 2005.

2.1.3 Utilization of soybeans

Soy food consumption patterns around the world varies widely, with such regions as Asia utilising soybeans primarily in such traditional foods such as tofu, soymilk and fermented products. In Western nations, soybeans are consumed in the form of refined soy protein ingredients (used in food processing) than in the production of tofu and soymilk. It is estimated that Asian nations utilise 95% of the soybeans consumed directly as human food in the world today. The consumption in Asia is based on long-standing traditional eating patterns and food

production methods whereas in Western nations, consumption of soybeans as direct human food is somewhat new phenomenon, although it is gaining increased acceptance and significance.

2.2 Overview of soyfoods in the market

Approximately 10% of the world's soybean crop is used directly for human food in which an array of products is made. Most of the soy-based foods utilise the whole soybean, while some are made with a variety of soy protein ingredients, including isolated soy protein, soy protein concentrate and soy flour. Out of a plethora of soyfoods currently available in the market including tofu, tempeh, miso, soysauce, okara, natto, soynuts as well as the cheese and cheese alternatives, only soymilk and soy yogurt will merit a detailed mention.

2.2.1 Soymilk

Traditionally, soymilk is the liquid extract of the soybean, which can be used in the preparation of tofu or as a nutritious beverage. However, the beverage quality of soymilk today has been improved using a number of modern food processing techniques to produce a blander product with greater appeal to Western tastes. Soymilk generally contains the most of the active phytochemicals present in soybeans, notably isoflavones. Some soymilks are made with soy protein isolate as a base. Many are fortified with vitamins A and D and calcium to bolster their position as a viable alternative to cow's milk. It can be put on cereal or made into yogurt, pudding, or ice cream.

2.2.2 Soymilk yogurt

Soymilk yogurt is made in the same manner as cow's milk yogurt. Pasteurised soymilk is inoculated with *L. acidophilus*, *Bifidobacterium*, or any other suitable cultures and incubated until the culture has turned the soymilk into yogurt. It tastes very similar to cow's milk yogurt and can

be presented in a variety of styles and flavours. It is very high in proteins and is a great source of isoflavones.

2.3 Drivers of market growth of soyfoods

The strong growth in the soy food market in the West has had a number of drivers which are briefly discussed below.

2.3.1 Technological innovations and “Westernisation” of soyfoods

Traditional soyfoods include soymilk, tofu, miso, natto and tempeh, the last three being fermented foods generally unattractive to Western tastes. Traditional soymilks made from whole beans have a strong “beany taste”, which is a positive attribute in some Asian countries, but generally negative in the West. Reduction of beany taste by, for example, the use of refined ingredients such as isolated soy protein, or by the use of proprietary taste masking or neutralising agents (Pszczola, 2000) has made soymilks much more acceptable to Western palates. New manufacturing processes and new soy ingredients have been developed to enable the manufacture of a broader range of products acceptable in the West, such as soy cheeses, soy yoghurts, soy breakfast cereals (Pszczola, 2000) and soy breads in which Australia led the world (Jorgensen *et al.* 1999). New processes have also allowed the production of a large range of meat alternatives more acceptable to the consumer. New soybean varieties with specific nutritional characteristics (eg high oleic acid, low saturated fat, low stachyose, low lipoxygenase) and new applications have also been or are being developed (American Soybean Association & United Soybean Board, 2001).

2.3.2 Incorporation of soyfoods into the product ranges of major food companies

The incorporation of soyfoods into the product ranges of large food companies has enabled major mass media marketing campaigns and increased consumer awareness as well as greater exposure

in major supermarket chains. These product categories include breakfast cereals, soy breads and dairy and meat substitutes. Linked to this has been the formation of a number of strategic alliances. For example, between Kraft Foods and Boca Burger which manufactures and markets soy-based meat alternatives in the US and between Dean Foods, a major US dairy company, and Silk a major soymilk manufacturer (Pszczola, 2000). In Australia, National Foods formed a joint venture with the Hong Kong company Vitasoy to manufacture soymilk in Australia (Spence, 2000). In the UK, Protein Technologies International, a major world soy protein company, has formed a joint venture with the Australian company Sanitarium aimed at building the European soymilk market (Mellentin & Heasman, 2001).

2.3.3 Consumer awareness of possible soy-health links

Another driver for market growth has been public interest in possible health benefits associated with consumption of soyfoods. Consumer surveys conducted for the US United Soybean Board report that the fraction of consumers who perceived soy and soy products as being healthy increased from 59% in 1997 to 69% in 2001 (National Report, 2002). Acceptance of a soy-health link was boosted in 1999 by approval by the USFDA of a health claim relating soy protein consumption to reduced risk of heart disease (Dotzel, 1999), and in 2002 UK authorities approved a similar claim (Gayer, 2002). In a similar vein, in 2000 the US Department of Agriculture, which had previously limited substitution of animal proteins by vegetable proteins (including soy) in the US Federal School Lunch Program to 30%, issued a ruling allowing their complete replacement and signalling recognition that soy protein was no longer considered a poor quality protein (Messina *et al.* 2002). To this extent, soy has moved from being considered merely a poor man's meat to be considered a true functional food. Whilst health claims are not yet permitted in Australia, soyfood manufacturers and ingredient suppliers have leveraged from the US claim to promote their products.

2.4 Phytoestrogens

Phytoestrogens occur naturally in many plants, and have structural and functional similarities to the human estrogen, 17β -estradiol (Cassidy, 1996). Other plant compounds with reported estrogenic properties include lignans, coumestans, and resorcylic acid lactones (Figure 2.1) (Knight & Eden, 1994). Mycoestrogens produced by fungi also have similar structures and effects. Phytoestrogens are classified according to their chemical structure and mainly fall into the class of flavonoids which include coumestans, lignans and isoflavones.

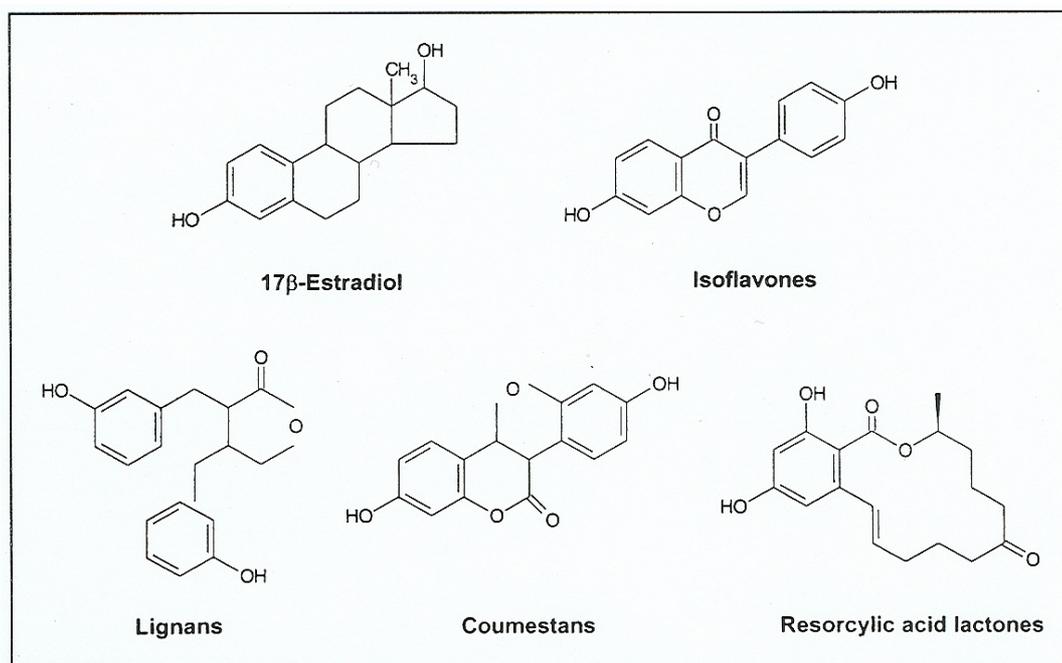


Figure 2.1 Chemical structures of the human estrogen, 17β -estradiol, and the classes of phytoestrogens compounds, isoflavones, lignans, coumestans, and resorcylic acid lactones

Lignans, though identified as a phytoestrogen, is not a flavonoid. They are found in a variety of plants including flax seeds, pumpkin seeds, sesame seeds, rye, soybeans, broccoli, beans, and some berries. When part of the human diet, lignans are converted into the mammalian lignans known as enterodiols (1) and enterolactones (2) by intestinal bacteria as shown in Figure 2.2.

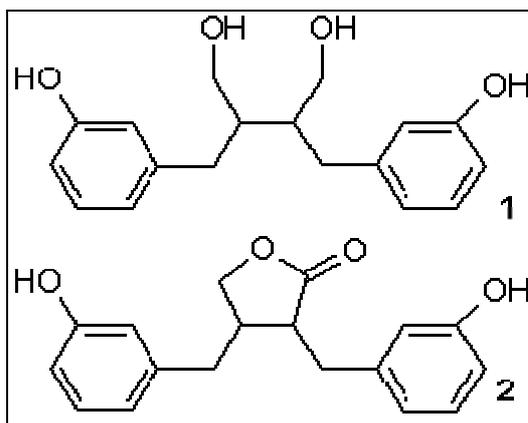


Figure 2.2 Chemical structure of mammalian lignan, enterodiol (1) and the metabolised intestinal lignan, enterolactone (2)

Coumestrol also has been identified as a soy isoflavone, although it is present at much lower concentrations in soybeans than are genistein, daidzein, and glycitein (Adlercreutz, 1998). Coumestrol, the estrogen mostly found in alfalfa, is the most potent of the plant estrogens. It attaches itself to sites in the brain and reproductive tract of animals. When consumed by young animals, coumestrol causes the uterus to grow and an increase in testicle length (Ososki & Kennelly, 2003).

2.4.1 Soy isoflavones

There are 4 chemical forms of isoflavones found in soybeans (Figure 2.3) namely malonyl-, acetyl-, β -glycoside conjugates and aglycones (King & Bignell, 2000). The biologically active, estrogen-like isoflavone compounds are the aglycone configuration of genistein, daidzein, and glycitein (Setchell & Cassidy, 1999). 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 was shown to yield its aglycone, genistein (Walter, 1941). Since then several investigators have shown the abundance of isoflavones in soybeans and other legumes. Studies have shown that there is a large variability in concentration and composition among different soybeans or soy-proteins products and that this is a function of species differences, geographic and environmental conditions, and the extent of industrial processing of

the soybean (Setchell *et al.* 1987a). Isoflavones of which there are approximately 230 individual types, are most commonly found in legumes,

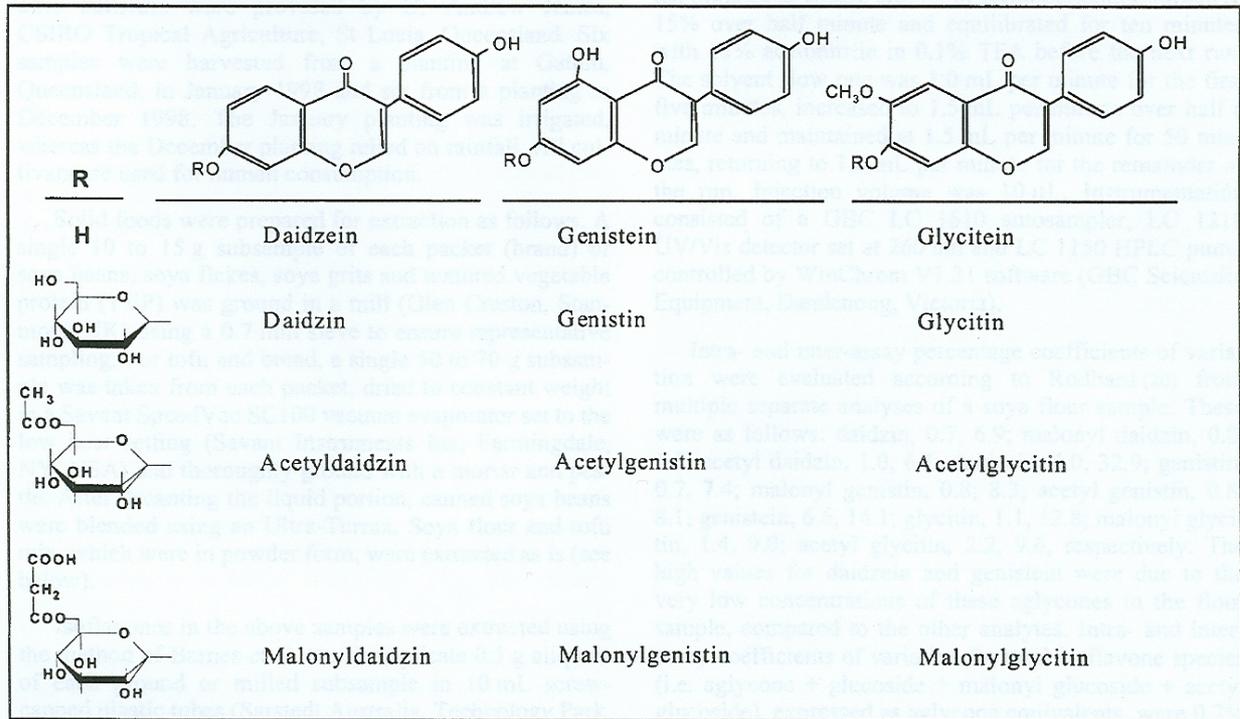


Figure 2.3 Four chemical forms of three analogues of isoflavones found in soybeans. Adapted from King & Bignell (2000).

with the highest amounts found in soybeans (Knight & Eden, 1994). Although there is a large variability of isoflavone composition among soybeans or soy-based food products, most dietary sources contain a mixture of derivatives based on three isoflavone aglycones with the common names genistein, daidzein, and glycitein (Figure 2.4) (Song *et al.* 1999). These three terms collectively describe all chemical forms of isoflavones that may be present in soy-based foods, in addition to the free phenolic or aglycone form, however, isoflavones exist as glycosides (Figure 2.5), acetylglycosides, or malonylglycosides.

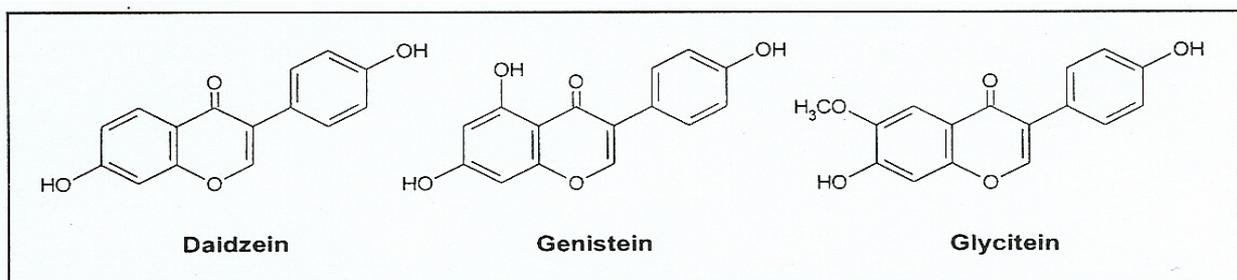


Figure 2.4 Chemical structures of the isoflavones, daidzein, genistein and glycitein, in aglycone form

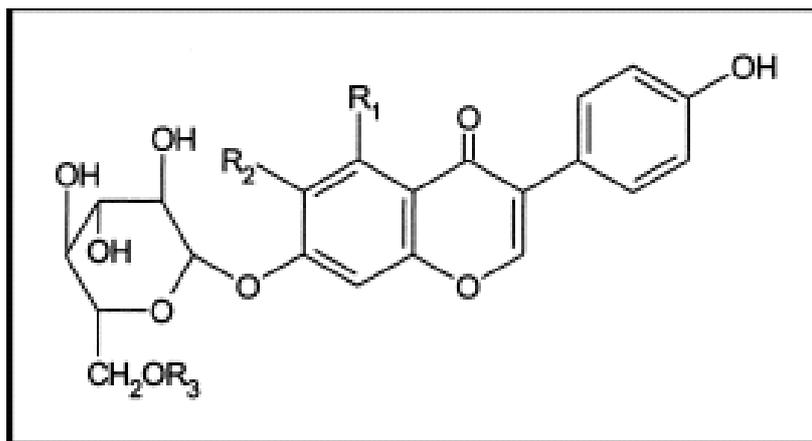


Figure 2.5 Chemical structure of a soy isoflavone glycoside.

2.4.2 Occurance of soy isoflavones

Although many varieties of vegetables, grains, and legumes contain small amounts of isoflavones, by far the largest quantities are found in soybeans, in which their content varies with variety, geographic location, soil type, and year and environmental conditions of growth (Wang & Murphy, 1994a). Processing, either through home preparation or within the food industry, also affects the amount and form of isoflavones in soy products (Reinli & Block, 1996). Generally, processing of soybeans for the manufacture of soy-containing food products increases the hydrolysis of isoflavone glycosides, resulting in higher concentrations of aglycones (Hutchins *et al.* 1995). One commonly consumed soy product is tofu, made by curdling fresh soymilk with a coagulant. Soymilk is produced by soaking finely ground soybeans in water. Other commonly consumed foods are miso, a paste used in soups and sauces made by aging soybeans with a grain-like rice for 1 to 3 years. Tempeh is made by fermenting soybeans with rice or millet. Natto, a topping for rice and vegetables is made by fermenting cooked whole soybeans and fried tofu. The fermentation of soybeans for products, such as tempeh and natto, will partially hydrolyze isoflavone glycosides giving them proportionally higher concentrations of aglycones (Hutchins *et al.* 1995). With the recent growth in vegetarianism, new soy products, such as soy burgers, soymilk, soy cheese slices, and soy yogurt, are gaining in popularity in Western countries.

Conversely, the "westernization" of Asian countries has contributed to a decrease in the consumption of traditional soy foods. In the few studies of isoflavone intakes in Western countries, average daily intakes are generally less than 2 mg isoflavones (de Kleijn *et al.* 2001). For subgroups that consume more soy-based foods than the general population, isoflavone intakes are closer to those of Asian countries. A study of Japanese Hawaiians indicated intakes of 12.5 mg isoflavones/day from tofu and miso, but other sources were not reported (Nomura *et al.* 1978). A more recent report from Hawaii compared women of different ethnic backgrounds attending a breast cancer clinic in Honolulu (Maskarinec *et al.* 1998). Intakes of both Chinese and Japanese women living in Hawaii were similar to their counterparts living in Asia, whereas native Hawaiians had lower, but still significant intakes. Caucasians and Filipinos had lower intakes, but these were still substantially higher than intakes in the United States or United Kingdom.

2.4.3 Metabolism of soy isoflavone

The biological activity and metabolic fate of dietary soy isoflavones differ depending on their chemical form (Cassidy, 1996). The chemical form of the isoflavone and its metabolites influence the extent of absorption, with aglycones more readily absorbed and more bioavailable than highly polar conjugated species (Setchell, 2000; Izumi *et al.* 2000). Following ingestion, the acetyl and malonyl derivatives of genistin and daidzin are metabolized to genistin and daidzin, which are then hydrolyzed in the large intestine by bacteria, resulting in the removal of the sugar moiety to produce their respective aglycones, daidzein, and genistein (Izumi *et al.* 2000). Following absorption of the aglycones, these compounds and their metabolites are readily conjugated in the liver with glucuronic acid and/or sulfate, circulate enterohepatically with potential metabolism and reabsorption in the intestine, and are excreted predominantly in the urine. Those isoflavones that are not absorbed are excreted in the unconjugated form in feces (Adlercreutz *et al.* 1995). The glucuronide fraction, the predominant conjugate, representing up to 90% of circulating isoflavones in both rats and humans, (Doerge *et al.* 2000) is considered

biologically inactive, (Cassidy, 1996) whereas the free and sulfated fractions, present at much lower concentrations, are generally thought to be biologically active. Alternatively, daidzein may be further metabolized by resident microflora in the gastrointestinal tract to equol and *O*-desmethylangolensin (ODMA) (via their respective intermediates, dehydroequol and dihydrodaidzein) (Figure 2.6) (Bayer *et al.* 2001). Similarly, genistein may be metabolized to 6'-hydroxy-*O*-desmethylangolensin via the intermediate dihydrogenistein (Figure 2.7), (Joannou *et al.* 1995) and further to p-ethyl-phenol. A recent *in vitro* study by Kulling *et al.* (2000) identified hydroxylated metabolites of both genistein and daidzein using liver microsomes from Aroclor-treated male Wistar rats, which the authors suggested might function as an important metabolic pathway *in vivo*. Metabolites of glycitein have recently been tentatively identified in human urine as 5'-OH-*O*-desmethylangolensin and 5'-methoxy-*O*-desmethylangolensin (Heinonen *et al.* 2000).

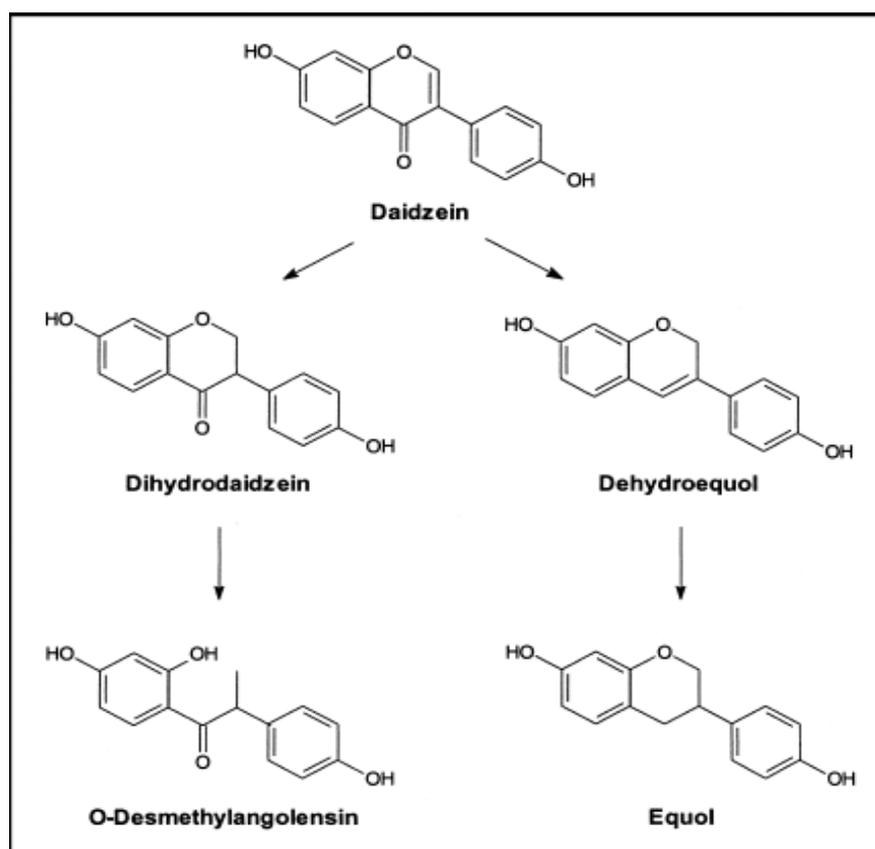


Figure 2.6 The metabolism of daidzein

The absorption, distribution, metabolism, and excretion (ADME) of soy isoflavones vary for subgroups in a population, based on age or gender, and among cultural groups. Interindividual variability has been documented in several studies (Xu *et al.* 2000) although evidence that

isoflavones may induce their own metabolism in some individuals is inconclusive. Investigation of variability in the ADME of isoflavones between males and females has produced inconsistent results (Lu & Anderson, 1998). Absorption and excretion of isoflavones have been reported in infants, with evidence that these may vary because the underdeveloped gut microflora cannot hydrolyze the glucuronide forms (Huggett *et al.* 1997). Additionally, differences in metabolic pathways may arise owing to different subpopulations of microflora, intestinal transit time, pH, or redox potential (Hendrich *et al.* 1999), factors that are influenced by diet, drugs (including antibiotics), bowel disease, surgery, and host immunity (Knight & Eden, 1995). Differences among cultural or geographic populations have not been systematically investigated, although it has been reported that some individuals are not able to form equol (Setchell & Adlercreutz, 1988).

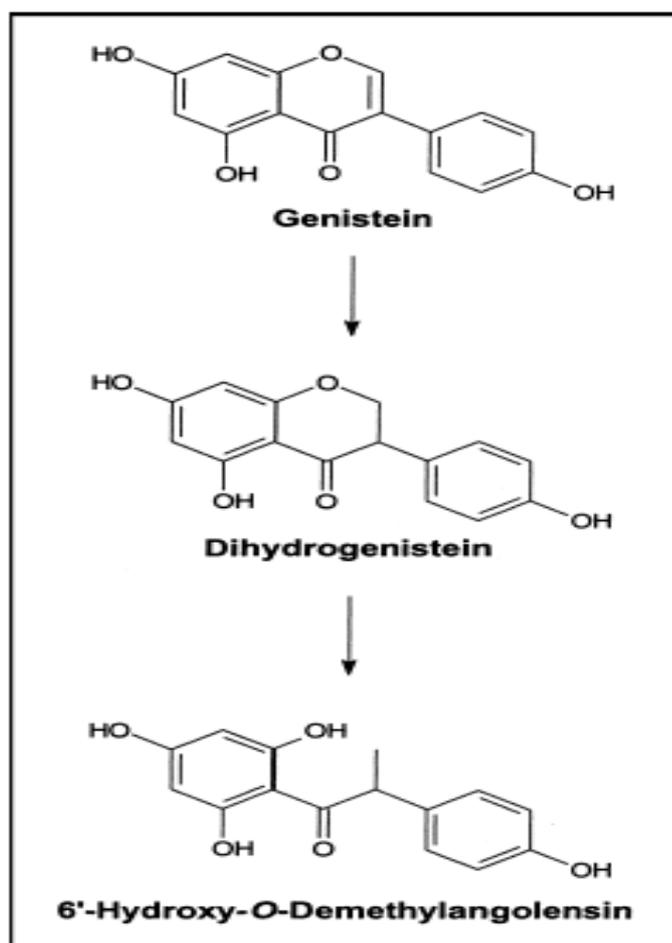


Figure 2.7 The metabolism of genistein

2.4.4 Summary of absorption and metabolic fate of isoflavones

It is beyond the scope of this section to summarise the present understanding of absorption and metabolism of isoflavones in detail. Briefly however, according to King (2002), the major points are, (i) absorption of isoflavones from the gastrointestinal tract occurs predominantly from the large bowel, although limited absorption also probably occurs from the distal small intestine, (ii) essentially no absorption of the isoflavones as intact glycosides occurs; they must be first hydrolysed to their aglycone forms by hydrolases in the intestinal wall or by enzymes of bacteria resident in the large bowel, (iii) during passage through the intestinal wall, as well as in the liver and other organs, the isoflavones are largely conjugated to form glucuronides and sulphates, (iv) isoflavones (mainly as glucuronides and sulphates) are excreted from the body mainly in urine, with only small amounts appearing in feces, (v) peak plasma concentrations of isoflavones appear approximately 6-9 hours after ingestion in humans, and essentially all are eliminated by 24-36 hours, (vi) extensive bacterial metabolism of isoflavones occurs in the large bowel; equol and *O*-desmethylangolensin are major metabolites, but a large number of other bacterial metabolites have been identified and the contribution of these (especially equol) to the biological effects of isoflavones is still a subject of considerable interest (Setchell *et al.* 2002b).

2.5 Health benefits of soy

Interest in the possible health benefits associated with eating soy foods has concentrated in three main areas - cardiovascular disease, cancer, and postmenopausal symptoms and consequences, although other diseases and conditions have been examined.

2.5.1 Cardiovascular disease

This is probably the area where there is strongest evidence for a health benefit. In October 1999 the Food and Drug Administration (FDA) in the USA accepted that there was sufficient evidence that soy foods reduce cholesterol for it to allow food manufacturers to label foods which meet

certain guidelines with a health claim to indicate that they may assist in reducing cardiovascular disease (Dotzel,1999). This was supported by an official statement from the American Heart Association (AHA) in 2000 (Erdman, 2000), and a similar health claim was approved in the UK in 2002 (Gayer, 2002). The FDA concluded that “based on the totality of the publicly available scientific evidence, that there is significant scientific agreement that soy protein, included at a level of 25 g/day in a diet low in saturated fat and cholesterol, can help reduce total and LDL-cholesterol levels, and that such reductions may reduce the risk of coronary heart disease (CHD)”. A cornerstone of the case for approval of this health claim was a meta-analysis of approximately forty relevant human studies published in 1995 (Anderson *et al.* 1995). This showed an ability of soy to significantly reduce LDL (“bad”) cholesterol in plasma, especially in individuals who had high initial levels. More recent well-controlled studies however have tended to show less reduction in LDL than those of the meta-analysis (Nestel , 2002). Interestingly, the FDA did not accept that the isoflavones in soy foods were directly involved in cholesterol reduction. Certainly, it appears clear that consumption of isoflavones alone does not reduce cholesterol (Howes *et al.* 2000). However, some have argued that whilst isoflavones alone are ineffective, there may be a synergistic effect of the soy protein or other components and the isoflavones (Clarkson, 2002). Distinct from effects on cholesterol, the effects of soy or isoflavones on a number of other processes or risk factors of relevance to cardiovascular disease have also been studied. For example, recent research, including some studies in Australia, have suggested that the isoflavones may assist in maintaining healthy arteries (van der Schouw *et al.* 2002; Yildirim *et al.* 2001; Walker *et al.* 2001) although not all studies have been able to demonstrate an effect. The effect of soy or isoflavones on blood pressure, has also been examined in humans. Some have provided support (Jenkins *et al.* 2002; Teede *et al.* 2001) whilst others have been unable to demonstrate a significant reduction (Hodgson *et al.* 1999), or have shown a slight increase under some circumstances (Hodgson *et al.* 1999). The reason for these differences is not clear, but does not seem to relate to differences in starting blood pressure. Oxidised LDL is

an important early initiator of formation of precursors of atherosclerotic plaques in the walls of blood vessels (Griffin, 1999). Substances that inhibit the oxidation of LDL may therefore protect against plaque formation. Many studies have shown the ability of genistein to inhibit oxidation of LDL in the test tube (Kerry & Abbey, 1998; Kapiotis *et al.* 1997), but concentrations employed are often much higher than are likely to be reached in the body. In order to obtain more physiologically relevant results, a number of studies have tested the ability of consumption of soy or isoflavone extracts by humans to inhibit LDL oxidation *ex vivo*. The results from these studies have been mixed. Some studies have been unable to demonstrate any beneficial changes (Samman *et al.* 1999), whereas others have demonstrated small, but statistically significant effects (Ashton *et al.* 2000; Scheiber *et al.* 2001). It is possible that the processes involved in isolating LDL from plasma in order to conduct the tests may lead in some cases to the loss of factors that may be operative *in vivo*. In order to overcome this difficulty, Hodgson *et al.* (1999) measured urinary excretion of the isoprostane 8-Epi-PGF2 α , a recently identified biomarker of free radical-induced lipid oxidation, following the daily consumption for eight weeks of a clover isoflavone supplement that supplied 55 mg isoflavones per day. They were unable to show any significant effect of the supplement. In contrast, Wiseman *et al.* (2000) showed that plasma 8-Epi-PGF2 α levels in subjects who consumed a soy diet high in isoflavones were 20% lower than when they consumed a soy diet low in isoflavones ($p < 0.05$), indicating protection of lipids (although not necessarily LDL lipids) from oxidation. Consistent with this finding, the conjugated diene content of LDL isolated from individuals consuming a soy diet was significantly lower than when consuming a control diet (Jenkins *et al.* 2000; 2002). There are a number of possible reasons for the different results, but it may be significant that Hodgson *et al.* (1999) used an isoflavone supplement, whereas the other studies used whole soy, suggesting the possible involvement of components other than the isoflavones.

2.5.2 Cancer and the risk of cancer

There is considerable evidence supporting a decreased risk of developing breast cancer in countries with increased consumption of soy-based foods (Wiseman, 1997). Cross-cultural studies have demonstrated lower mortality from breast cancer in Japan than in the United States (Kelsey & Horn, 1993). For example, and case-control studies suggest that breast cancer patients have lower soy intake than the control (Ingram *et al.* 1997). In an epidemiologic study, Wu *et al.* (1996) correlated increased risk of breast cancer with low intakes of soy in Asian-Americans and determined that soy intake was correlated with birthplace; people born in Asia consumed more soy than those of Asian descent born in the United States. In addition, soy intake decreased as the number of years of residence in the United States increased (Wu *et al.* 1996). In a study of the effect of several putative factors on the incidence of breast cancer in Singaporean women, a decreased risk was reported in premenopausal women as the proportion of soy protein in total dietary protein increased (Lee *et al.* 1992); no such correlation was found for postmenopausal women. The authors hypothesized that the dual effect of soy protein consumption was hormone mediated because it was more strongly associated with menopausal status than age. The results of a study by McMichael-Phillips *et al.* (1998) indicate a positive association between soy consumption and an increase in the proliferation of breast lobular epithelium in premenopausal women; however, these results were not reproducible by Hargreaves *et al.* (1999) under the same conditions. Additionally, *in vitro* studies found stimulation of growth of human breast cancer cell lines at low, but physiologically relevant concentrations of genistein, but inhibition of growth at higher concentrations (Zava & Duwe, 1997). In the presence of physiologic doses of both genistein and estrogen, the former was reported to competitively inhibit estrogen binding and slightly inhibit cellular proliferation (de la Rochefordiere *et al.* 2001).

In an attempt to elucidate the mechanisms of action of soy isoflavones on breast epithelial proliferation, Lamartiniere (2000) investigated mammary gland morphology and cell differentiation in rat. The author concluded that neonatal treatment with genistein resulted in

early mammary gland development, with more terminal end buds in 21-day-old rats compared with controls, thus confirming the proliferative effects of soy isoflavones (Lamartiniere, 2000). No difference in the size of the mammary gland of the treated rats versus controls at 50 days of age was apparent, however, and fewer terminal end buds and more lobules, i.e., a more differentiated terminal ductal structure, was reported in rats treated neonatally or prepubertally with genistein. Lamartiniere (2000) concluded that early exposure to genistein enhances cell differentiation of the mammary gland, and may confer a protective effect against carcinogenesis via this process. Furthermore, *in vitro* studies using cultured human breast cancer cells indicate that genistein inhibited the growth of both estrogen receptor-negative and estrogen receptor-positive cell lines (Wang & Kurzer, 1997), and induced the activity of two phase II detoxification enzymes, NAD(P)H: quinone reductase and glutathione-S-transferase (Pahk & DeLong, 1998). Genistein may therefore exert anticarcinogenic effects by enhancing the detoxification of carcinogens.

In a case-control study, Ingram *et al.* (1997) compared the diet and urinary phytoestrogen concentration of women recently diagnosed with breast cancer with well-matched controls, and reported that women with breast cancer had significantly lower urinary concentrations of the isoflavone, equol, and the lignan, enterolactone, further supporting earlier studies suggesting that dietary phytoestrogens may be protective for breast cancer (Ingram *et al.* 1997). However, Duncan *et al.* (2000) noted that the relationship between equol excretion and a decreased risk for breast cancer may be due in part to the hormonal profile of equol excretors, compared with non-equol excretors. High equol production may stimulate SHBG production, similar to the effects of genistein (Pino *et al.* 2000), which has approximately the same estrogenic activity. High SHBG concentrations lower the percentage of free estradiol in plasma and subsequently reduce the biologic activity of estradiol (Adlercreutz *et al.* 1995).

To evaluate the hypothesis that consumption of an isoflavone-containing diet may alter the metabolism of 17β -estradiol, Lu *et al.* (2000) evaluated the urinary excretion of two estrogen

metabolites over a period of one menstrual cycle in eight pre-menopausal women consuming a diet containing genistein and daidzein (70-125 mg total aglycone/day) compared with an isoflavone-free diet. In addition to an increase in the excretion of genistein and daidzein during the isoflavone-rich diet period, the authors reported increased excretion of 2-hydroxyestrone (a proposed anticarcinogenic metabolite) (Bradlow *et al.* 1994) and no change in the excretion of 16 α -hydroxyestrone (a putative carcinogenic metabolite) (Adlercreutz *et al.* 1994). The increase in the ratio of urinary 2-hydroxyestrone to 16 α -hydroxyestrone during the isoflavone-rich diet period was suggested to be a possible contributing factor to the lowering of serum 17 β -estradiol concentrations and a reduced long-term risk for breast cancer (Lu *et al.* 2000). The increased 2-hydroxyestrone to 16 α -hydroxyestrone ratio as a mechanism for the decrease in serum 17 β -estradiol concentrations has not been confirmed and effects may be attributable to gonadotropin concentrations (Adlercreutz *et al.* 1995). The majority of studies investigating the relationship between soybean isoflavones and endometrial cancer have been epidemiologic, and have generally concluded that increased soy intake reduces the risk of developing endometrial cancer. Goodman *et al.* (1997) examined several lifestyle factors, including diet, in 341 women with endometrial cancer, and in 511 controls that were matched for ethnicity (Hawaiian, Japanese, Caucasian, Native Hawaiian, Filipino, and Chinese) and age. An inverse relationship between consumption of soy products and risk of endometrial cancer was found for each ethnic group. This result is consistent with epidemiologic data, which shows that there is a lower incidence of endometrial cancer in Asian populations compared with North American and European populations (Dhom, 1991).

In *in vitro* studies, genistein inhibited the growth of a cultured human prostate cancer cell line, possibly through inhibition of focal adhesion kinase (Kyle *et al.* 1997), or through interaction (Boersma *et al.* 2001) with halogenated or nitrated oxidants, resulting in modified biologic activities of the isoflavone. Morton *et al.* (1997) compared the concentration of daidzein and equol in the prostatic fluid of European men with that of Asian men. The concentrations of

daidzein and equol in the prostatic fluid of men from Hong Kong were significantly higher than concentrations in men from Portugal and Great Britain. This is consistent with previous studies of urinary and plasma isoflavone concentrations and epidemiologic data regarding the frequency of consumption of soy products (Dhom, 1991). The correlation between countries with a low incidence of prostate cancer and either high soy consumption or high physiologic isoflavone concentrations is consistent with a protective effect of soybean isoflavones on the prostate. Vegetarians or those consuming soy products in Western countries also have a lower risk of prostate cancer, although the importance of increased isoflavone intake in any chemoprotective effect is not known relative to possible effects of other dietary components (Morton *et al.* 1997). In summary, studies of soy foods and breast cancer therefore fall into four main categories. The first involve comparisons of the incidence of breast cancer in different countries that have differences in the consumption of soy foods (eg. Japan compared to Australia), as well as comparisons between high and low soy consumers within countries. Some of these epidemiological studies suggest that soy foods may be protective (Jakes *et al.* 2002; Wu *et al.* 2002; Dai *et al.* 2002), but not all studies support a link (den Tonkelaar *et al.* 2001; Horn-Ross *et al.* 2001). There is also evidence that exposure to isoflavones before puberty is important to endow a protective effect (Wu *et al.* 2002; Lamartiniere *et al.* 1998a) and that protection may be more effective premenopausally than postmenopausally (Lee *et al.* 1992). The second type of studies have involved the use of animals in which cancers are induced by some means and the incidence of tumours in those that were fed soy is compared to animals that were fed a control non-soy diet. These studies generally suggest that soy and isoflavones are protective (Gallo *et al.* 2001; Constantinou *et al.* 2001), although this is not always the case as is shown by findings from other studies (Allred *et al.* 2001; Cohen *et al.* 2000). The third type of studies involve the use of cancer cells grown in test tubes in which the effect of isoflavones on their growth is tested. These types of studies generally show a biphasic effect. At genistein concentrations above about 10 μ M inhibition of growth has been uniformly reported; however at lower concentrations, around 1 μ M,

(which are closer to plasma concentrations found in soy consumers *in vivo*) slight stimulation of growth occurs under some conditions (Messina & Loprinzi, 2001). The final type of studies involve administration of isoflavones or soy to women and the determination of the effect on biomarkers that may relate to cancer risk, and some of these have shown increased levels of risk markers in nipple aspirate (Hargreaves *et al.* 1999) and biopsy samples (McMichael-Phillips *et al.* 1998). The cautionary results from some of these studies have led to concerns in some quarters, particularly regarding use of isoflavone supplements (as apposed to soyfoods), and particularly with regard to use by breast cancer sufferers (de Lemos, 2002). In summary, the outcomes of studies that have examined the link between soy or isoflavones and breast cancer risk are inconclusive, and any relationship can only be proved with long-term prospective studies in humans.

2.5.3 Post-menopausal symptoms

The consequences of menopause include hot flushes, night sweats, vaginal dryness, mood swings and osteoporosis and they affect approximately 80-85% of postmenopausal women in Western countries and can be quite debilitating (Eden, 2001). Conventional estrogen replacement therapy (HRT) is very effective, leading to a reduction of about 70% in hot flushes (Elkind-Hirsch, 2001). However, for a number of reasons, including concerns about cancer, many women are seeking alternative, or so-called “natural”, therapies to treat menopausal symptoms. Soy and isoflavones are one class of natural therapy and there have been a number of studies to determine their efficacy, particularly with regard to hot flushes.

2.5.3.1 Hot flushes

In a consensus statement published in 2000 (Greenwood *et al.* 2000) based on review of nine studies (two reported in abstract form) published at that time, the North American Menopause Society concluded that with regard to hot flushes the effect of soy and isoflavones was inconclusive. There is however on going research in this area and more findings will be reported

when conclusive reports are available. Dosages of isoflavones ranged from 30 to 150 mg per day, and period of treatment from 4 to 12 weeks. Most studies have been double blind placebo controlled and two have been multi-centre studies (Upmalis *et al.* 2000; Faure *et al.* 2002). Uncertainty and controversy however remain (Davis, 2001; Husband , 2002) and it is clear that more research will be required to resolve this uncertainty.

2.5.3.2 Osteoporosis

Osteoporosis is a serious and potentially debilitating consequence of the menopause in many women, particularly in the West (Cooper *et al.* 1992). Whilst HRT is effective in preventing bone loss in early menopause, there may be side effects (Morabito *et al.* 2002) and as with other menopausal symptoms women are therefore seeking other treatments for this condition. A synthetic isoflavone, ipriflavone (which interestingly gives rise to the soy isoflavone daidzein as one of a number of intermediates upon metabolism) has been studied for at least three decades (Gennari, 1997). It has been marketed since the late 1980s in at least twenty countries for the clinical treatment of osteoporosis (Gennari, 1997) with generally positive results (Maugeri *et al.* 1994). Doses are high, in the range 500 - 1000 mg/d (Maugeri *et al.* 1994) and whilst these are not achievable for isoflavones from soy foods, they may be relevant if daidzein is a major active metabolite of ipriflavone. Studies of the effects of soy and isoflavones have used *in vitro* methods, animal models and human studies (Anderson & Garner, 1998). The animal studies have generally used ovariectomised rodents (Ohta *et al.* 2002; Uesugi *et al.* 2001), or in some cases ovariectomised primates (Lees *et al.* 1998) and these have provided some supportive evidence, however isoflavone doses are often in the range 5-50 mg/Kg body weight per day, much higher than could be achieved in humans by normal dietary intake. The human studies have generally involved postmenopausal women and have been epidemiological (Nagata *et al.* 2002; Greendale *et al.* 2002), as well as clinical intervention (Uesugi *et al.* 2002; Chiechi *et al.* 2002) in design and have used bone mineral density and/or urinary or plasma markers of bone metabolism as end

points. They have been conducted in both Western (Clifton-Bligh *et al.* 2001) and Asian countries (Uesugi *et al.* 2002). The results have been mixed – some have provided support (Greendale *et al.* 2002) whilst others have not (Chiechi *et al.* 2002). The reasons for these differences are not clear, but is perhaps not altogether surprising when study times have ranged from a few weeks to several years; isoflavone and soy doses have varied, the sites for testing bone mineral density have varied, as have the biomarkers measured. The North American Menopause Society concluded that at the time of their review there was insufficient evidence about effects of isoflavones on bone health to draw firm conclusions (Clifton-Bligh *et al.* 2001).

2.5.3.3 Estrogen replacement therapy

Estrogen replacement therapy (ERT) has long been used in postmenopausal women to aid in treating the symptoms of menopause, such as hot flushes and sweats, as well as a possible preventative treatment for bone loss and cardiovascular disease. Because of structural similarities of phytoestrogens to endogenous estrogen, the possibility for use of phytoestrogens, like soy isoflavones, as an alternative to traditional ERT for the treatment of menopausal symptoms has been investigated, but overall, the results are equivocal. In a 12-week study, Murkies *et al.* (1995) investigated the effect of a soy-supplemented diet on reduction of menopausal symptoms in 23 postmenopausal women consuming 45 g soy flour/day (providing approximately 60 to 90 mg aglycone isoflavones, calculated using the USDA-Iowa State University database on the isoflavone content of foods composition data for soy flour). For women consuming the soy-supplemented diet, a significant increase in urinary daidzein excretion over the 12-week period was reported, accompanied by a rapid decrease in the number of hot flushes in the first 6 weeks, and a total reduction of 40% in the number of hot flushes in the 12 weeks of the study. Additionally, consumption of a soy extract (providing 50 mg total aglycone isoflavones per person per day) alone or in combination with ERT in the form of conjugated equine estrogens, was reported to decrease the number of hot flushes in postmenopausal women with no reported

effects on vaginal cytology, endometrial thickness, uterine artery pulsatility index, or other metabolic and hormonal parameters tested (Scambia *et al.* 2000). Contrary to these results, Quella *et al.* (2000) reported that soy isoflavones were not effective in treating menopausal symptoms in a study of 177 breast cancer survivors. A conclusion to the effectiveness of soy isoflavones as a replacement for ERT has not yet been reached; however, no soy isoflavone-related toxicity has been reported in any of these studies.

2.5.3.4 Cardiovascular function

Postmenopausal women are at increased risk for cardiovascular disease because many risk factors are aggravated by menopause. Phytoestrogens may modulate risk factors favourably, involving mechanisms similar to estrogen. Cardiovascular disease (CVD) is the leading cause of death among women in Westernized societies (Mosca *et al.* 1997). Cardiovascular disease risk increases strongly after menopause due to diminished estrogen production (Barrett, 1997). Several risk factors are influenced by menopause, including a decrease in serum HDL cholesterol (Matthews *et al.* 2001), changes in plasma clotting and fibrinolytic factors (Gebara *et al.* 1995), and an increase in plasma total homocysteine (Hak *et al.* 2000). Homocysteine is a nonprotein amino acid of methionine metabolism, and an elevated plasma concentration is considered to be a strong and independent risk factor for cardiovascular disease in epidemiologic studies (Welch & Loscalzo, 1998).

In a study of 31 hyperlipidemic volunteers (19 men and 12 postmenopausal women) consuming a soy-containing diet providing 86 mg glycoside isoflavones/day (53.4 mg total aglycone) (Jenkins *et al.* 2000), decreased concentrations of circulating oxidized LDL were reported, with no evidence of increased urinary estrogenic activity. The diet was consumed for two 1-month periods separated by a washout period of 2 weeks. Teede *et al.* (2001) reported that when healthy men and postmenopausal women received a soy protein isolate in beverage form that provided 118 mg glycoside isoflavones per day (73.3 mg total aglycone) or a placebo for a period of 3

months, reduced systolic, diastolic, and mean blood pressure and lowered triacylglycerol and LDL/HDL cholesterol concentrations were observed in volunteers receiving the soy beverages. The authors also reported a decrease in mean brachial artery flow-mediated vasodilation (FMD) and an increase in Lp(a) lipoprotein blood concentrations. Teede *et al.* (2001) stated that a rise in Lp(a) lipoprotein may be a cofactor rather than an independent risk factor for cardiovascular disease, and noted that the FMD assay has limitations of poor repeatability, significant operator dependence, and influence of external factors such as exercise and diet. No effects on the hypothalamic-pituitary-testicular axis were observed. In a study by Tsangalis (2004), 5 of 13 women at postmenopausal stage consuming soymilk that provided 80 mg isoflavones per day for 12 weeks, the women consuming fermented soymilk showed a significant increase in HDL-cholesterol levels compared to the casein-milk group, but were not accompanied by reductions in total cholesterol, LDL-cholesterol and triglyceride.

2.5.4 Cognitive function and neurologic diseases

The effect of soy consumption on cognitive function was assessed in elderly subjects enrolled in the Honolulu Heart Program cohort of the Honolulu-Asia Aging Study (White *et al.* 1996). The diet of subjects in this group was assessed between 1965 and 1972, and their cognitive abilities assessed in 1991 to 1993 when the subjects were between 71 and 93 years of age. An association between tofu consumption in midlife and reduced cognitive function and structural changes of the brain was reported in these Japanese-American males (White *et al.* 1996, 2000). However, in a review of this hypothesis, the FDA stated "If tofu or soy were implicated in Alzheimer's disease, its prevalence would be expected to be higher in Japan than in Hawaii, but White *et al.* (1996) found the prevalence of Alzheimer's disease to be higher in Hawaii than in Japan." Whereas a cohort study of Seattle Japanese-American men and women indicated that consumers of high amounts of tofu had lower cognitive function scores at baseline, tofu consumption was not associated with any change in cognitive function over 2 years of follow up (Rice *et al.* 1999).

Interpretation of these preliminary results should be tempered by the observational nature of the study designs and the potential for the mediation of the effects by confounding factors (Grodstein *et al.* 2000). Moreover, these results were not replicated in a short-term dietary intervention study, in which it was reported that consumption of a high-soy diet (100 mg total isoflavones per day or 62 mg aglycone per day) versus a low-soy diet (0.5 mg total isoflavones per day) for a period of 10 weeks did not affect attention and improved memory, cognitive function, or mood in healthy male and female student volunteers (File *et al.* 2001). Cross-cultural studies have not detected significant differences in dementia between Asian and Western populations (Graves *et al.* 1994; Yoshitake *et al.* 1995). Epidemiologic studies of the incidence of dementia in Japan compared with Western countries suggest that high intakes of soy isoflavones do not promote neurological disease (Graves *et al.* 1994). The prevalence of dementia in persons over age 65 has been estimated to be between 3.5 and 10.3% in the United States and between 4 and 7% in Japan. However, the composition of dementia subtypes varies between these populations. Alzheimer's disease accounts for the majority (50-70% or more) of the total number of dementia cases in the United States and Europe, whereas in Japan, vascular diseases cause the majority (30-60%) of dementia cases. The reasons for the difference in predominant subtype is not known, but may result from differences in lifestyle, culture, environment, genetic factors, risk of stroke, or diagnostic criteria for dementia. (Grodstein *et al.* 2000; Graves *et al.* 1994).

2.5.5 Bone development

Bone mass decreases with increasing age and this decrease is due to increased bone resorption and reduced bone formation. The decrease in bone mass induces osteoporosis, which is widely recognized as a major public health problem. The most dramatic expression of this disease is represented by fractures of the proximal femur (Cooper & Melton, 1992). Recent studies have shown that daidzein and genistein, natural isoflavonoid phytoestrogens found in *Leguminosae*, have an anabolic effect on bone metabolism in rats (Yamaguchi *et al.* 2000). The oral

administration of genistein (50 g to 100 g) to young and elderly rats for 14 d caused a significant increase in the femoral dry weight, calcium content, alkaline phosphatase activity, and DNA content in the diaphyseal and metaphyseal tissue, indicating that the isoflavone has an anabolic effect on bone components in aged rats. Genistein has been shown to stimulate a cell proliferation due to increasing protein synthesis in osteoblastic cells (Ono & Yamaguchi, 1999). Genistein may stimulate osteoblastic bone formation and mineralization in the femoral-diaphyseal and metaphyseal tissues of young and elderly rats. Genistein has also been shown to inhibit osteoclastic bone resorption (Gao & Yamaguchi, 1999). Bone resorption in aged rats may be inhibited by the administration of genistein, thereby increasing bone calcium content.

2.6 Profiling of isoflavones in commercial soy bean foods

The conjugated forms of isoflavones present in a soy nutritional supplements (predominately acetyl glycosides) and in blood from two human volunteers after consuming the supplement (7- and 4-glucuronides and sulfates) have been identified using liquid chromatography coupled with electrospray/tandem mass spectrometry. The soy isoflavones consist of genistein, daidzein, and, to a lesser extent, glycitein, and total isoflavones are in the range of 0.1 to 3 mg/g d.wt. The isoflavones in soybeans were previously characterized chromatographically by using liquid chromatography (LC) with UV and mass spectrometric detection (Barnes *et al.* 1994; Wang & Murphy, 1994a,b). Wang and Murphy (1994a,b) reported the isoflavone content and composition of American and Japanese soybeans in Iowa in different crop years at different growth locations and found that crop year and location both impacted the isoflavone contents. They also reported that Japanese soybeans had different profiles from the American soybeans. Wang and Murphy (1994a,b) also reported that Japanese soybeans had higher ratios of 6''-O-malonyl isoflavones to Glycosides than did American soybeans. In another study, Wang and Murphy (1994a), showed that the contents of commercial soybean food products were affected by the variety of soybean, the processing, and the dilution with non soy ingredients. During formulation of second

generation soyfoods as opposed to the traditional soyfoods, modification such as hydrolysis of the glycosides occurs thus altering the profile of isoflavones in the product. Thus analytical procedures such as HPLC, LC-MS and / or both are have been used in determining the isoflavone content of various soy foods. These procedures have been used to identify several glycoside conjugates, primarily the malonyl esters, in addition to trace amounts of the aglycones in soymilk and are reported in Chapter 8.0 of this thesis.

2.6.1 Isoflavone content of soy food products in Australian market

Isoflavone levels in foods available in the Australian Market are scant and not all isoflavones have been quantified. Dalais *et al.* (1997) reported the isoflavone contents of a range of soy and linseed breads, soymilks and various other soy products such as tofu, miso, soy flour and soy sauce found in a local supermarket in Melbourne, Australia. The isoflavones reported were however not conclusive as only daidzin, genistin and their aglycones daidzein and genistein were quantified using high performance liquid chromatography (HPLC). The levels of isoflavones varied from one food product to the other and given their potential benefit in human diet, regular analysis of new products is needed to ascertain the quantity that would guarantee adequate levels of these compounds (Dalais *et al.* 1997). Table 2.2 show the isoflavone contents of some of the soy based foods found in the local safeway supermarket in Melbourne, Australia.

Table 2.2 Isoflavone levels in some of the foods in an Australian Supermarket

Food	Daidzin mg/g or mL	Genistin mg/g or mL	Daidzein mg/g or mL	Genistein mg/g or mL	Total mg/g or mL
Soybread	0.049	0.071	0.007	0.007	0.134
Soymilk	0.024	0.058	0.002	0.005	0.089
Soyflour	0.894	1.400	0.004	0.007	2.305
Tofu	0.112	0.144	0.013	0.013	0.282
Miso	0.053	0.124	0.058	0.071	0.306
Soysauce	ND	ND	0.009	0.004	0.013

ND – Not Detected

Part of the challenge facing researchers is to establish isoflavone doses that produce either beneficial or toxic responses in humans. In the modelling and processing of foods with functional attributes particularly those containing bioactive compounds like isoflavones, it is important to characterise the milieu of the bioactive compounds, monitoring the changes at every processing step and how the composition can be influenced during processing to attain the desired composition and concentrations that would guarantee health benefits on consumption. Thus part of the goals of this study was to develop an analytical methodology to characterize the existing isoflavones in the soymilk made from SPI SUPRO 590 in order to regulate doses that would confer health benefits of the soymilk. Details of profiling of the isoflavones using extracted ion chromatography and positive ion fragmentation techniques are detailed in Chapter 8.0 of this thesis.

2.7 The stability of soy isoflavones during storage and processing

The bioavailability and bioactivity of many phytochemicals is affected by their chemical structure. For soy isoflavones, these changes in their chemical structure are very likely to happen during storage and processing (Shimoni, 2004). Davies *et al.* (1998) showed that soy protein isolate (SPI) underwent browning during storage for more than 2 years at room temperature and that this browning reaction was parallel to genistein loss thereby showing that long term storage might lead to the loss of aglycone genistein. During processing however, Singletary *et al.* (2000) showed that extrusion of soy/corn mixture at 120°C resulted into an average loss of 24% of the isoflavones. Indeed, the recent work of Ungar *et al.* (2003) showed the degradation of daidzein and genistein in model solutions altered the antioxidant potential of the solution. Interestingly, the changes in antioxidant potential were different for daidzein and genistein, and depend on the pH. At pH 7, both isoflavones lost only a minor part of their antioxidant potential, whereas at pH 9, genistein lost most of the antioxidant activity and daidzein products maintained most of their potency. The authors concluded that daidzein is more labile to thermal treatments than genistein, and that its degradation products have higher antioxidant activity. It is therefore possible that

these 2 isoflavones have a different degradation mechanism. Furthermore, changes in the isoflavones profile may modify the biological benefits of the specific food. We report in Chapters 5.0, 6.0 and 7.0 a comprehensive evaluation on the stability of isoflavone isomers in soymilk during storage.

2.7.1 Processing and storage-induced changes in soy isoflavones

The concentration and the composition of soy isoflavones in soy foods and soy-containing foods vary markedly. These differences may result from variability in formulation, from changes induced by the raw material being used and the processing methods and techniques, post processing changes during distribution and storage. These changes may occur as early as during the storage of the raw materials. Long-term storage of 15 soybean cultivars showed minor decrease in the total isoflavones concentration over a period of 1 to 3 y of storage (Lee *et al.* 2003). However, the concentrations 6-*O*-malonyldaidzin and 6-*O*-malonylgenistin decreased dramatically during the same storage period. Similar decrease in the malonyl derivatives was also reported for soybean seeds stored at 80°C and was accompanied by the formation of acetyls and glycosides (Kudou *et al.* 1991). Hence, the malonyl derivatives appear to be most labile in the soybean raw material. The effects of processing techniques on the distribution of isoflavones were also investigated in the manufacturing of tempeh, soymilk, tofu, and protein isolate (Wang *et al.* 1998). Wang and Murphy (1996) found that the manufacturing steps causing significant losses of isoflavones were 12% during soaking and heat processing (49%) in tempeh production, 44% during coagulation in tofu processing, and 53% during alkaline extraction in soy protein isolate (SPI) production. Malonyldaidzin and malonylgenistin decreased after soaking and cooking in the production of tempeh, soymilk, and tofu. Concomitantly, acetyldaidzin and acetylgenistin were generated during heat processing. Alkaline extractions in protein isolate processing caused the generation of daidzein and genistein, probably through alkaline hydrolysis. The preparation of SPI resulted in a loss of 20% of the isoflavones found in the soy flour (Wang *et al.* 1998). Processing significantly affected the retention and distribution of isoflavones in

food. A recent study examined genistein and daidzein stability in aqueous media at conditions simulating commercial sterilization processes (Ungar *et al.* 2003). The stability of genistein and daidzein was studied at 120°C in alkaline (pH, 9) and neutral environments (pH, 7). A difference in the stability of genistein and daidzein was detected, whereas in alkaline solution, genistein concentration was reduced by 60%, only a minor 15% reduction was observed for daidzein. At neutral pH, daidzein was less stable than genistein and its concentration decreased by 40%, compared with 22% for genistein. High temperature and pressure reduced the total isoflavones content in corn-soy mixtures (Singletary *et al.* 2000). The study of Mahungu *et al.* (1999) on extrusion at high temperatures (110°C, 130°C, and 150°C) reported a decrease in the overall isoflavones content of SPI and corn mixtures. In addition, in their study, the loss of the daidzein and its conjugates (44%) was higher than that of genistein (33%). As in the study of Lee *et al.* (2003), most of isoflavone degradation in the study of Mahungu *et al.* (1999) was attributed to loss of the malonyl derivatives. The degradation and modification of soy isoflavones are not limited to high water content products such as soymilk, tofu, and tempeh. Coward *et al.* (1998) studied the chemical modification of isoflavones in soy foods during cooking and processing. Analysis of soy food products revealed that defatted soy flour that had not been heat-treated consisted mostly of malonyl conjugates. In contrast, toasted soy flour contained many 6-*O*-acetyl- β -glycoside conjugates formed by heat-induced decarboxylation of the malonate group to acetate. Baking or frying of textured vegetable protein at 190°C and baking of soy flour in cookies did not alter total isoflavone content, but there was a steady increase in β -glycoside conjugates at the expense of malonyl conjugates. The stabilities of the 3 isoflavones at different heating temperatures in their dehydrated form were investigated by Xu *et al.* (2002). Daidzin, glycitin, and genistin lost 26%, 27%, and 27% of their original concentration, respectively, after 3 min at 185°C. Heating at temperatures above 135°C produced acetylated daidzin and genistin, daidzein, glycitein, and genistein. The rate of formation of acetyl derivatives was higher than the rate of loss of a glycoside group to form daidzein and genistein. They also found that the stability of

daidzein was higher than that of glycitein or genistein. Changes in isoflavones composition and content may also occur during storage of soy products. An interesting report by Hayes *et al.* (2001) brings evidence for changes in soy isoflavones after ultra high-temperature processing of chocolate beverages. There was a decrease in the malonyl derivatives and a continuous change in the isoflavone profile within the various families. They also pointed out that these changes continued during storage and were affected by storage temperature. Eisen *et al.* (2003) followed the changes in isoflavones content during storage of soy milk. A decrease in genistin content was observed at elevated temperatures, and also during 6 month storage experiments at ambient conditions. The mechanism by which isoflavones are lost is somewhat obscure. Wang *et al.* (1990) reported that when standard genistein was mixed with dextrose, fructose, maltose, and sucrose, it formed conjugates with very high ultraviolet (UV) absorption. The amount of these conjugates formed was proportional to the amount of added sugar. Another potential route for isoflavone degradation in composite solutions was reported by Davies *et al.* (1998), suggesting that isoflavones can react in Maillard-type reactions. These experiments were performed in model systems. Because Maillard reaction products are known to be potential carcinogens, undesirable products may be produced in this reaction.

2.7.2 Degradation kinetics of soy isoflavones

The ability to predict the impact of specific processing and storage conditions on isoflavones content and composition is crucial for optimization of processes. This requires obtaining reliable data on the kinetics of their degradation under various environmental conditions. Table 2.3 summarizes the available kinetic data. In the study of Davies *et al.* (1998), using model genistein solutions (pH, 9), genistein concentration decreased rapidly and reached 50% of the initial concentration after less than 1 wk of incubation at 60°C. Grun *et al.* (2001) studied the changes in isoflavones composition during thermal treatment of tofu. Assuming first order kinetics, total isoflavones degradation during 40 min at 80°C, 90°C, and 100°C revealed kinetic constants of 5

to 13 (1/d). During this thermal treatment, genistein derivatives were only slightly affected, whereas most of the isoflavone decomposition was due to loss of free daidzein, which may be more labile to thermal treatments than genistein. The kinetics of genistin loss in soymilk was determined by following its concentration in soymilk samples stored at various temperatures (Eisen *et al.* 2003). The decrease in genistin concentration appeared to be of first order kinetics, as shown in Figure 2.8, for the degradation of genistin in soymilk stored at 90°C. Similar degradation pattern was found at ambient temperature ranging from 15°C to 37°C. Rate

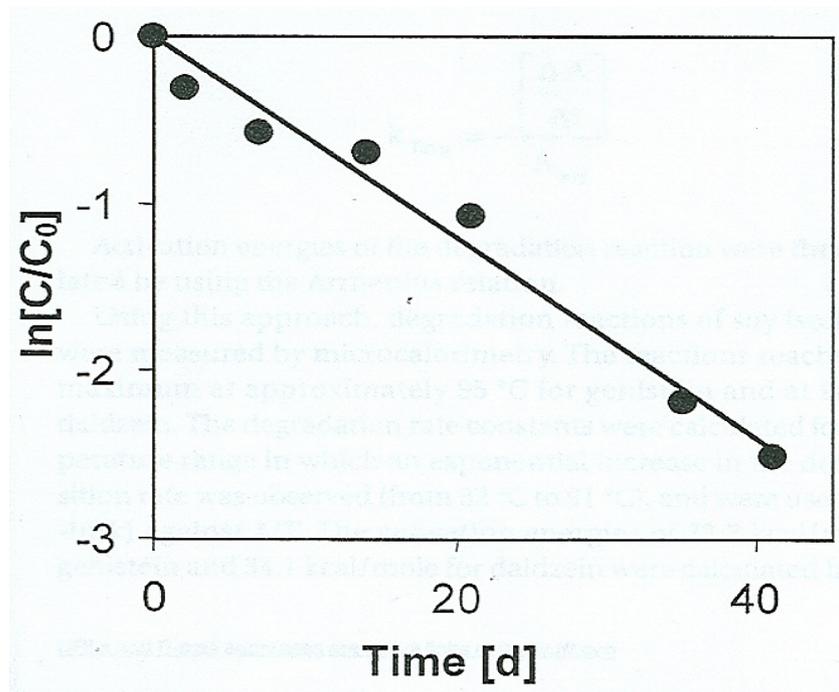


Figure 2.8 Genistin degradation in soymilk stored at 90°C. Adapted from Eisen *et al.* 2003

constants were calculated assuming first order kinetics for genistin degradation in all incubation temperatures. At the higher temperature range, the reaction rate constants were found to be up to 3 orders of magnitude higher than at ambient temperatures. A follow-up study by Ungar *et al.* (2003) characterized the kinetics of daidzein and genistein degradation in model solutions (pH, 7 and 9). The results of the kinetic experiments reaffirm that the degradation reaction is apparently a first order reaction. Genistein degradation was accelerated in an alkaline environment. For example, the rate was 0.222 (1/d) ($P < 0.001$) for 90°C at pH 9 and only 0.030 (1/d) ($P < 0.001$) at pH 7. Higher stability at pH 7 was also detected in daidzein solutions. Overall, the degradation

rates of daidzein were higher than for genistein, demonstrating again that daidzein is indeed more labile to degradation than genistein at high temperatures. In light of the scarce data, the elucidation of isoflavone conversion or degradation during processing and storage remains an unsolved problem. For instance, during heating, malonylgenistin can be converted to acetylgenistin or genistin. In addition, acetylgenistin can be converted to genistin or genistein, depending on heating temperature and time. Moreover, in addition to conversion, all these isoflavones may undergo degradation simultaneously. Thus, the conversion and degradation mechanism of isoflavones during heating is an extremely complicated phenomenon. For appropriate prediction, the rate constants for each conversion and degradation should be determined separately. Although simple first order equations may fit the observed degradation, these are far from a true description of the degradation kinetics.

2.7.3 Effects of temperature on degradation of isoflavones

The temperature dependence of soy isoflavones degradation appears to follow the Arrhenius relation. Meta analysis of the data for total isoflavones loss during thermal treatment of tofu resulted in an activation energy (E_a) for daidzein degradation of 19.5 kcal/mol (Grun *et al.* 2001). This higher sensitivity of daidzein loss rate to temperature was also shown by results where its loss rate was the highest among isoflavones in tofu. In soymilk, the calculated activation energies for genistin loss at elevated and ambient temperatures were 17.6 and 7.2 kcal/mol, respectively (Eisen *et al.* 2003). These lower activation energy values may indicate some type of oxidation reaction. In addition, the difference in the apparent activation energies at high and low temperatures suggests that a number of reactions take place at the same time. In this case, the reaction may become dominant at elevated temperatures and thus change the apparent those of the measured degradation rate. The activation energies for daidzein degradation reported by Ungar *et al.* (2003) in a model system were similar to activation energies reported previously for the degradation of epigallocatechin gallate (Zimeri & Tong, 1999). The E_a for the catechins was

18.7 kcal/mol as compared with 8.38 kcal/mol and 21.69 kcal/mol for pH 9 and pH 7 for daidzein, respectively.

Table 2.3 Kinetic parameters for degradation of soy isoflavones in different products at different temperatures

Isoflavone	Product	Temp (°C)	Order	Rate Constant	Reference
Genistein	pH 9 buffer (Tris 0.4 M)	60	Zero	8.65×10^{-2} (mmole/l/d)	Davies <i>et al.</i> 1998
Daidzein	Tofu	100	First	47 (1/d)	Grun <i>et al.</i> (2001)
Malonyldaidzin	Tofu	100	First	56 (1/d)	Grun <i>et al.</i> (2001)
Genistin	Soymilk	15	First	0.0004 (1/d)	Eisen <i>et al.</i> (2003)
		25	First	0.0011 (1/d)	
		37	First	0.0039 (1/d)	
		70	First	0.0611 (1/d)	
		80	First	0.0778 (1/d)	
Genistein	pH 9 buffer (borate 0.1 M)	90	First	0.1092 (1/d)	Ungar <i>et al.</i> (2003)
		90	First	0.222 (1/d)	
		80	First	0.102 (1/d)	
		70	First	0.087 (1/d)	
		90	First	0.030 (1/d)	
Daidzein	pH 7 buffer (phosphate 0.1M)	80	First	0.025 (1/d)	
		70	First	0.022 (1/d)	
		90	First	0.547 (1/d)	
		80	First	0.323 (1/d)	
		70	First	0.277 (1/d)	
Daidzein	pH 9 buffer (borate 0.1 M)	90	First	0.262 (1/d)	
		80	First	0.091 (1/d)	
		70	First	0.045 (1/d)	

Adapted from Shimoni (2004)

Determining the temperature dependence of soy isoflavones degradation is a laborious and long process, mainly due to the low rate constants. Thus, there is an interest in alternative fast stability testing methods. One potential method is to use the microcalorimetric stability testing (Ungar *et al.* 2003), commonly used for drugs and pharmaceuticals. Differential scanning calorimetry (DSC) microcalorimeter was used to evaluate the stability of the isoflavones and to assess the enthalpies of the degradation reactions. These were used to calculate the kinetic constants and

activation energies for each reaction. Samples were scanned by temperature ranging from 50°C to 120°C at a scanning rate of 3.8°C/h, followed by a rescan. The heat capacities (C_p) were obtained for every temperature (kcal/mol°C), and the enthalpies (H) of the degradation reactions were calculated. The degradation rate constants were calculated for a temperature range, in which exponential increase in the decomposition rate was observed.

Cumulative evidence from various studies indicates that soy isoflavones may be modified and degraded in the raw material during processing and storage of the final product. Although the apparent rate of the degradation reactions appears to be slow, the fact that soy isoflavones are key bioactive compounds may be important in terms of the definition of “end of shelf life” for soy products. Labuza (2000), noted that the structure-function benefit that is used to attract the consumer is based on some active agent. It would be therefore required for the manufacturer to know the effect of processing, distribution, and storage on this active agent in the finished product. The stability of bioactive compounds in food has a much wider scope than just soy isoflavones. The possibility that a bioactive chemical might be involved in reactions that alter its biological activity should be taken into consideration. It is even more important when such a compound is part of a food, sold as a functional food or a dietary supplement. In a period when much attention is given to the biological benefits of natural bioactive food components, the question addressing their stability has been sometimes overlooked (Shimoni, 2004). To the best of our knowledge, there have been no studies conducted previously in the assessment of the stability of individual isoflavone isomers in soymilk during storage. Details of findings on isoflavone degradation during storage are found in Chapters 5.0, 6.0 and 7.0. of this thesis.

2.8 Probiotic Bacteria

The word “probiotic”, originating from the greek work “for life”, is used to classify viable microorganisms that demonstrate beneficial effects on the health of the host upon ingestion by improving the balance of microflora in the gut (Fuller, 1989). The primary probiotic bacteria

associated with dairy products include *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium*. However, health benefits imparted by probiotic bacteria are strain-specific and not species- or genus specific (Playne, 2002). Probiotic bacteria are typically ingested via fermented dairy products such as yogurt and fermented soymilk, soy foods (e.g. soy yogurt) or freeze-dried probiotic capsules. To obtain the desired therapeutic effects of probiotic bacteria, it has been suggested that these microorganisms be present in food at high levels of $\geq 10^6$ viable cells per gram, to compensate for the possible reduction in numbers during the passage through the stomach and the intestine (Shah, 2000).

It is a common practise to combine two or more probiotic strains or a combination of probiotic and starter culture strains in the manufacture of probiotic foods, thereby influencing the viability and therapeutic efficacy of probiotic bacteria (Shah, 2000). On the whole, the potential health benefits associated with the ingestion of these probiotic bacteria include treatment and prevention of rotaviral-, traveller's-, antibiotic-associated- and *Clostridium difficile*-diarrhoea, lessening of lactose intolerance, reduction of constipation, modulation of immune response, alleviation of atopic dermatitis symptoms in children, and elimination of *Helicobacter pylori* (organism responsible for gastric ulcers) (Isolauri *et al.* 2001). Furthermore, Playne (2002) indicated further that there is emerging evidence to indicate that probiotic bacteria may play a role in the prevention of bowel and bladder cancer, irritable bowel syndrome and inflammatory bowel diseases (e.g. Crohn's disease), and arthritis. In addition, St-Onge *et al.* (2000) stated that evidence from animal and human studies suggests a moderate cholesterol-lowering effect associated with the consumption of fermented dairy products containing probiotic strains, possibly reducing the risk of cardiovascular disease.

2.8.1 Genus *Lactobacillus*

The genus *Lactobacillus* is by far the largest of the genera included in lactic acid bacteria (Axelsson, 1998). It is therefore very heterogenous, encompassing species with large variety of

phenotypic, biochemical and physiological properties. Table 2.4 shows a summary of the characters used to distinguish between the three main subgroups within this genus and some more well known species included in each group.

Table 2.4 Arrangement of the Genus *Lactobacillus*

Character	Group I Obligately homofermentative	Group II Facultatively heterofermentative	Group III Obligately heterofermentative
Pentose fermentation	—	+	+
CO ₂ from glucose	—	—	+
CO ₂ from gluconate	—	+ ^a	+ ^a
FDP aldolase present	+	+	—
Phosphoketolase present	—	+ ^b	+
	<i>Lb. acidophilus</i>	<i>Lb. casei</i>	<i>Lb. brevis</i>
	<i>Lb. delbrückii</i>	<i>Lb. curatus</i>	<i>Lb. buchneri</i>
	<i>Lb. helveticus</i>	<i>Lb. plantarum</i>	<i>Lb. fermentum</i>
	<i>Lb. salivarius</i>	<i>Lb. sake</i>	<i>Lb. reuteri</i>

^aWhen fermented

^bInducible by pentoses

Adapted from Sharpe (1981) and Kandler and Weiss (1986)

2.8.1.1 *Lactobacillus acidophilus*

Lactobacillus acidophilus is one of several bacteria in the genus *Lactobacillus*. It is commonly used commercially together with *Streptococcus salivarius* in the production of yogurt. *Lactobacillus acidophilus* gets its name from *lacto-* meaning milk, *-bacillus* meaning rod-like in shape, and *acidophilus* meaning acid-loving. This bacterium thrives in more acidic environments than most microorganisms (pH 4-5 or lower) and grow best at 45°C. *L. acidophilus* occurs naturally in a variety of foods, including dairy, grain, meat, and fish. It is also present in human (and animal) intestines, mouths, and penises. *L. acidophilus* (and all lactic acid bacteria) absorb lactose and metabolize it into lactic acid. Certain related species (known as heterofermentative) also produce ethanol, carbon dioxide, and acetic acid this way. *L. acidophilus* itself (a homofermentative microorganism) produces only lactic acid. Like many bacteria, *L. acidophilus* can be killed by excess heat, moisture, or direct sunlight.

2.8.1.2 Health benefits of *Lactobacillus acidophilus*

L. acidophilus is considered a probiotic or "friendly" bacterium. These types of healthy bacteria inhabit the intestines and vagina and protect against some unhealthy organisms. The breakdown of nutrients by *L. acidophilus* produces lactic acid, hydrogen peroxide, and other by-products that make the environment hostile for undesired organisms. *L. acidophilus* also tends to consume the nutrients many other microorganisms depend on, thus outcompeting possibly harmful bacteria in the digestive tract. During digestion, *L. acidophilus* also assists in the production of niacin, folic acid, and pyridoxine. Studies have also shown *L. acidophilus* can assist in bile deconjugation, separating amino acids from bile acids, which can then be recycled by the body.

Constipation is a common problem in subjects consuming the Western diet and also in the elderly. Reported benefits of alleviating constipation using *L. acidophilus* NCFB 1748 (Rajala *et al.* 1988), *L. casei* Shirota (Ogawa *et al.* 1974). Additional reports confirm that *L. acidophilus* provides relief from indigestion and diarrhea. The same strains have also been found to be successful in alleviating enteric infections. The beneficial effects of lactobacilli has been attributed to their ability to suppress the growth of pathogens, probably by secretion of antibacterial substances such as lactic acid and bacteriocins (McCormic & Savage, 1983). *L. acidophilus* have also been found to have anti-microbial effects against pathogens and fungal microorganisms (Buttris, 1997). Since *L. acidophilus* is able to survive in environments of pH 4-5 or below, it is able to survive the harsh conditions of the stomach and pass through to the small intestine. Some research has indicated *L. acidophilus* may provide additional health benefits, including improved gastrointestinal function, a boosted immune system, and a decrease in the frequency of vaginal yeast infections. *L. acidophilus* is part of the normal vaginal flora. The acid produced by *L. acidophilus* in the vagina helps to control the growth of the fungus *Candida albicans*, helping to prevent vaginal yeast infections. The same beneficial effect has been observed in cases of oral or gastrointestinal Candidiasis infections. Also Gilliland & Walker, (1990) reported that an *L. acidophilus* strain NCFM had an appreciable ability to assimilate

cholesterol and may be useful for lowering plasma cholesterol in humans resulting in the reduced risk of coronary heart disease in persons with high blood serum cholesterol levels.

2.8.1.3 *Lactobacillus acidophilus* and Food Production

L. acidophilus can be used to make yogurt, sweet acidophilus milk, and other fermented dairy products. The bacteria produce lactate and acetate during fermentation. In yogurt production, *L. acidophilus* is added to milk to render it more acidic. The lactic acid it produces causes milk proteins to break down, coalescing into a more nearly solid gel substance - yogurt. By breaking down lactose to lactic acid, the bacteria make such dairy products digestible by lactose intolerant people. It can also be used to make "cultured soy" (commonly called soy yogurt) from soymilk in much the same way.

2.8.2 *Lactobacillus casei*

Lactobacillus casei is a transient, anaerobic microorganism of genus *Lactobacillus* found in the human intestine and mouth. As a lactic acid producer, it has been found to assist in the propagation of desirable bacteria. This particular species of *Lactobacillus* is documented to have a wide pH and temperature range, and complements the growth of *L. acidophilus*, a producer of the enzyme amylase (a carbohydrate-digesting enzyme). It is known to improve digestion and reduce milk intolerance and constipation. The most common application of *L. casei* is industrial, specifically for dairy production. They are also reported to be successful in lowering amounts of the compounds causing flatulence upon digestion such as raffinose in soybeans. As a result, some strains have been studied along with *L. acidophilus* and *Bifidobacterium* in the fermentation of soymilk and their specific role in isoflavone biotransformation reported in Chapters 3.0, 4.0, 7.0, 9.0 and 10.0

2.8.3 Genus *Bifidobacterium*

Ever since their first isolation from faeces of breastfed infants in 1899 by Tissier of the Pasteur Institute, the genus *Bifidobacterium* has gradually evolved to include up to 31 species to date.

Eleven of these species have been isolated from humans (adults and/or infants), and the remainder from intestinal tracts or rumen of animals (Tannock, 1999). Other known sources of *Bifidobacterium* species include honey-bees (Shah & Lankaputhra, 2002), wastewater and fermented milk (Gomes & Malcata, 1999). Presently, five species of *Bifidobacterium* have attracted the attention in the dairy industry for manufacturing probiotic milk products; *B. bifidum* (most commonly used), *B. breve*, *B. infantis* and *B. longum* (Shah & Lankaputhra, 2002), all of which have been isolated from humans.

Bifidobacteria are the predominant members of the human gastrointestinal microflora, especially in the ileum and colon where they are found at populations of 10^3 to 10^7 and 10^8 to 10^{12} CFU per gram of intestinal contents, respectively Orrhage & Nord, 2000; Shah & Lankaputhra, 2002). When available in sufficient numbers in the intestinal tract, bifidobacteria create a healthy equilibrium between beneficial and potentially harmful microorganisms (Shah & Lankaputhra, 2002).

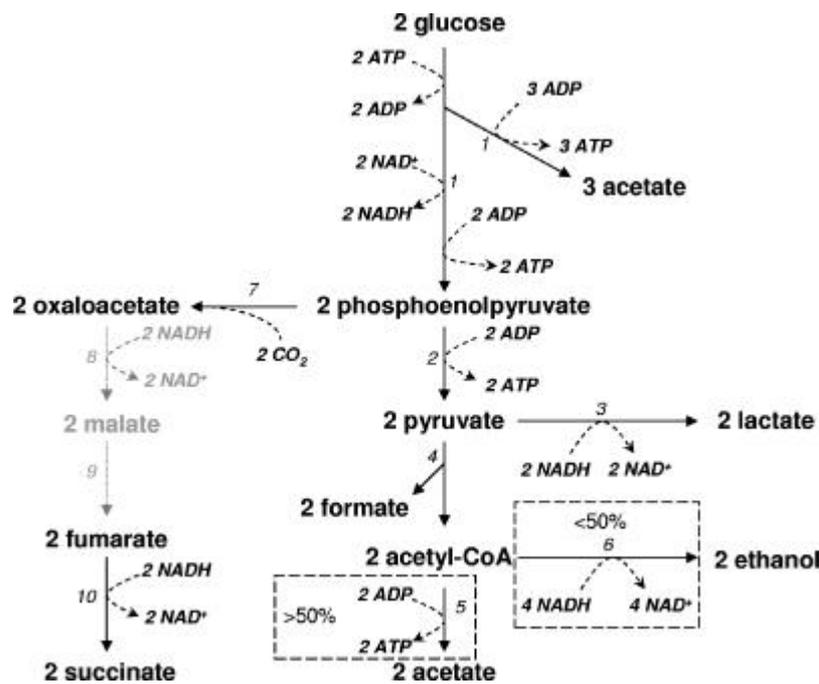


Figure 2.9 Schematic diagram of bifidobacterial sugar metabolism. 1, Enzymes of the bifidobacterial fructose-6-phosphate shunt; 2, pyruvate kinase (*pyk*); 3, lactate dehydrogenase (*ldh*); 4, pyruvate formate lyase (*pfl*); 5, phosphotransacetylase (*pta*) and acetate kinase (*ack*); 6, acetaldehyde dehydrogenase (*adh*) and alcohol dehydrogenase (*adh*); 7, phosphoenolpyruvate carboxylase (*ppc*); 8, malate dehydrogenase (coding sequence not found in either *B. longum* genome); 9, fumarase (coding sequence not found in either *B. longum* genome); 10, succinate dehydrogenase (*sdh*). The values in boxes are the percentages of conversion of acetyl-CoA.

Bacteria of the genus *Bifidobacterium* present a globally bacillar form, showing Gram- positive staining, are non motile, non-spore forming, and catalase-negative anaerobes. They have various shapes including short, curved rods, club-shaped rods and bifurcated Y-shaped rods (Ballongue, 1993). They are saccharolytic organisms that exclusively degrade hexoses by the fructose-6-phosphate pathway (Scordovi & Trovatelli, 1965), with the fermentation of two moles of glucose generally leading to the production of three moles of acetate and two moles of lactate without generation of CO₂ (Figure 2.9). The main enzyme involved in this glycolytic pathway is fructose-6-phosphate phosphoketolase (F6PPK), which is used as a taxonomic character in identification of the genus (Gomes & Malcata, 1999). Besides glucose, all bifidobacteria from human origin are able to utilise galactose and lactose as carbon sources (Krzewinski *et al.* 1996; Gomes & Malcata, 1999). Additionally, studies have shown that *Bifidobacterium* are able to metabolise complex carbohydrates amylose and amylopectin and oligosaccharides raffinose and stachyose (Scalabrini *et al.* 1998), potentially utilising their monomers as growth substrate. They also produce numerous intracellular and extracellular saccharolytic enzymes during growth, including β -glucosidase, β -galactosidase, α -galactosidase and α -glucosidase (Tochikura *et al.* 1986; Desjardins & Roy, 1990). The optimum growth temperature of bifidobacteria is 6.0 to 7.0, with virtually no growth at pH 4.5 to 5.0 or below or at pH 8.0 to 8.5 or above (Gomes & Malcata, 1999). As for temperature, bifidobacteria can grow in the range of 25 to 45°C, with the optimum growth temperature being 36 and 38°C for strains of human origin and 41 to 43°C for those of animal origin (Ballongue, 1993). Growth of bifidobacteria does not occur below 20°C; hence, refrigerated storage effectively maintains the viable populations of bifidobacteria attained through fermentation.

Some of the most commercially available strains that have most published clinical data on their potential health benefits include *Lactobacillus paracasei* Shirota (Yakult) and *Bifidobacterium animalis* ssp. *lactis* Bb12 (formerly known as *B. lactis* Bb12; Chr Hansen). Due to the production of numerous intracellular and extracellular enzymes, *Bifidobacterium* has found wide application in the fermentation including processes involving hydrolysis of glycosides in soymilk (Tsangalis *et al.* 2002, 2003). Due to their involvement in improving the biological activity of soymilk,

further studies on the stability of the hydrolysing enzymes at various storage temperatures have been conducted and reported in Chapter 3.0, and evaluation of the enzymic potential of *B. animalis* ssp *lactis* Bb12 in isoflavone transformation reported in Chapter 4.0.

2.8.4 Metabolic activity of probiotic bacteria in soymilk

Soybean contains oligosaccharides such as raffinose and stachyose that are not digested by human beings and may cause flatulence (Wang *et al.* 2002). This drawback along with the disagreeable bean flavors has often limited the consumptions of soybean and some soy based foods. To overcome these limitations and to develop a probiotic dietary adjunct, the fermentation of soymilk has been studied with the probiotic cultures of lactic acid bacteria and bifidobacteria (Wang *et al.* 2002). It was reported that soymilk could support the simultaneous growth of *Lactobacillus acidophilus* CCRC 14079 or *Streptococcus thermophilus* CCRC 14085 with *Bifidobacterium infantis* CCRC 14633 or *Bifidobacterium longum* B6 (Wang *et al.* 2002). Besides, it was found that a significantly higher reduction in the contents of stachyose and raffinose, with the increase in the contents of sucrose, fructose, galactose plus galactose in soymilk could be achieved through the fermentation with mixed cultures of bifidobacteria and lactic acid bacteria (Wang *et al.* 2002). These observations fuelled the possibility and the potential of developing the lactic acid bacteria and bifidobacteria-containing probiotic soymilk dietary adjunct. But an additional reason to engage the use of probiotic bacteria in the fermentation of soymilk is the occurrence of endogenous β -glucosidase which hydrolyses the predominant isoflavone glycosides into bioactive isoflavone aglycones. Building on previous findings by Tochikura *et al.* (1986), it has been demonstrated throughout this study that endogenous enzymes (β -glucosidase and β -galactosidase) from probiotic bacteria can be used to hydrolyse the predominant isoflavone glycosides in soymilk to improve the biological activity of soymilk by increasing the concentration of bioactive isoflavone aglycones. This creates an exciting prospect for modelling functional soy based foods that contain high levels of bioactive isoflavone aglycones, with optimum aglycone equivalence as well as high viable counts ($\geq 10^6$ viable cells per gram) of probiotic bacteria as outlined in Chapters 9.0 and 10.0. There is also an

extensive report on the influence the two hydrolysing enzymes in the degradation of individual isomers of isoflavones in soymilk during storage at different temperatures in Chapters 5.0, 6.0 and 7.0. Due to the health benefits of isoflavones already outlined, the use of probiotic bacteria to increase bioactive isoflavone aglycone concentration is discussed throughout the thesis (Chapters 3.0 – 11.0).

2.9 Challenges with soy isoflavone-based nutritional supplements

The presence in soybeans of genistein and its glycoside, genistin, has been recognized for many decades. For much of that time it was believed that the primary form of this isoflavone in unprocessed soybeans was the glycoside form, genistin. Similarly, daidzin and its aglycone daidzein have long been known as soy constituents. There is now a large literature describing the biological properties of genistein in particular, because of its activity as a tyrosine kinase inhibitor, possible anti-oxidant and potential anti-cancer compound, among other notable discoveries. Over the years much has also been published regarding the interesting and potentially useful activities of daidzein and daidzin. More recently, another glycoside/aglycone pair, namely glycitin and glycitein respectively, were identified in soy extracts. Because of the rather low levels of glycitin and glycitein in soy products and attendant lack of affordable material for experimental use, their biological properties have remained largely a mystery in spite of their potential importance. Because the malonyl and acetyl glycosides have only recently been found in soybean products, their specific biological, pharmacological and nutritional properties are largely unknown at the present time. Given the increasing use of soy products and the many beneficial nutritional properties attributed to the isoflavone components of those soy products, it appears that further studies of the biological role of these novel soy isoflavones are of substantial scientific and medical interest.

It has been claimed that some nutritional supplements in the market, containing certain proportions of the soybean isoflavones and intended for human use, may be of low activity or may even have adverse effects, for the following reasons. Epidemiological studies comparing Asian and Caucasian diets and disease rates, and dietary trials using soybean products,

isoflavones or isoflavone concentrates, have established with a reasonable degree of certainty that "soy isoflavones" have several useful biological activities in humans. The vast majority of these studies over the years involved bulk forms of soy or soy extracts containing the typical natural ratios of genistein/daidzein/glycitein-based components of approximately 1.3/1.0/0.2 (expressed as the aglycone equivalent). Indeed, much attention has focused on genistein alone, and daidzein has been found to be nearly inactive in many *in vitro* assays. However, there are soy isoflavone supplements in the market containing dramatically different ratios of the individual isoflavone equivalents. For example, the Solgar company has sold "Super Concentrated Isoflavones" having a genistein/daidzein/glycitein ratio of 0.33/1.2/1.0 in a recent lot (5 mg, 18 mg and 15 mg/capsule, respectively), and the Source Naturals company has sold a product it calls "Genistein" with a ratio of 0.4/1.7/1.0 in a recent lot (8 mg genistein, 34 mg daidzein and 20 mg glycitein per capsule). These products have genistein/glycitein ratios differing respectively by 20- and 16-fold from the natural ratios in bulk dietary soybean products and extracts upon which most of the human nutritional science to date has been based. Such products contain very little genistein equivalent but have large amounts of daidzein equivalent, thought to be largely inactive, and glycitein equivalent, whose effects in humans at these high, unnatural doses are presently unknown. Thus it appears that these products may be largely ineffective at inducing their intended biological activities. This depends on the as-yet-untested effects in humans of high doses of glycitein when ingested in the absence of the 6.5-fold larger amounts of genistein normally consumed along with glycitein in bulk soy foods. Soy-based nutritional supplements such as the Solgar, Source Naturals and other products constitute a large, growing and heavily promoted component of the nutritional supplement industry. With such widespread use, research directed at obtaining a better understanding of the properties of their constituents appears to be of considerable importance. In Chapter 10.0, we demonstrate how the bioactive isoflavone aglycone concentrations can be increased using probiotic microorganisms as well as exogenous β -glucosidase enzyme while concurrently monitoring the aglycone equivalent during processing. The two types of products that can be processed, can be a significant starting point for clinical studies to verify their therapeutic potential.

3.0 Stability of β -glucosidase Activity Produced by *Bifidobacterium* and *Lactobacillus* spp. in Fermented Soymilk during Processing and Storage

3.1 INTRODUCTION

The term probiotic was derived from the Greek, meaning “for life”. The Food and Agriculture Organisation of the United Nations (FAO) and World Health Organisation (WHO) have stated that there is an adequate scientific evidence to indicate that there is potential for probiotic foods to provide health benefits and that specific strains are safe for human use (FAO & WHO, 2001). The expert panel commissioned by FAO and WHO defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. Probiotics are normally marketed as capsules and powders or added to yoghurts or yogurt-like products. The diverse range of ‘functional’ or ‘pharma’ food products currently available reflects the convenience of using food as a delivery system for probiotic microorganisms (Driessen & de Boer, 1989). However, certain criteria need to be fulfilled to ensure the production of high quality foods that maximise their biotherapeutic potential. These include the incorporation of sufficient numbers of microorganisms into the product, the maintenance of viable populations during shelf-life of the food and subsequent survival of these microbes through the gastro-intestinal tract in order to attain intact delivery to the colon which is the usual site of action (Berrada *et al.* 1991; Sanders *et al.* 1996).

Lactobacillus acidophilus, *bifidobacterium*, and *Lactobacillus casei* are among the predominant members of the intestinal microflora. There is an increasing evidence to suggest that some members of the lactic acid bacteria, such as *bifidobacterium* and lactobacilli, when consumed in sufficiently large numbers exhibit prophylactic and therapeutic properties in both humans and animals (Mitsuoka, 1990). Due to their potential health benefits, selected strains of

bifidobacterium are widely used in dairy preparations in conjunction with probiotic bacterium *L. acidophilus* (Shah, 2000).

L. acidophilus, *bifidobacterium*, and *L. casei* grow slowly in soymilk during product manufacture. Therefore, the established practice is to incorporate yogurt cultures (i.e. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) along with probiotic cultures. It is reasonable to assume that the beneficial effects of probiotic bacteria can be expected only when viable cells are ingested (Shah, 2000). Furthermore, the enzymic activity within the cells is dependent on the viability of the microorganisms. Probiotic microorganisms possess β -glucosidase, β -galactosidase and α -galactosidase (Tochikura *et al.* 1986), which play an important role in the hydrolysis isoflavone glycosides to the bioavailable aglycones forms.

Commercial β -glucosidases have been used in biotransformation of isoflavone glycosides (Park *et al.* 2002, 2003) and β -glucosidase from *bifidobacterium* have been used in hydrolysing the β -1,6 Glycoside bonds in order to increase the concentration of bioactive isoflavone aglycones in soymilk (Tsangalis *et al.* 2002, 2003). However, the stability of the enzyme has never been studied and the impact of stability on isoflavone biotransformation during storage remains unknown. Therefore the objectives of this study were to screen strains of *L. acidophilus*, *bifidobacterium*, and *L. casei* for optimal β -glucosidase activity in the soymilk and examine the stability of the enzyme derived from selected strains of the three groups of microorganisms during storage at different temperatures (-80°C, 4°C, 25°C and 37°C) for 8 weeks.

3.2 MATERIALS AND METHODS

3.2.1 Bacteria

Pure cultures of *L. casei* ASCC 292, 1520, 2607, ASCC 290, 279, ASCC 1521, *L. acidophilus* ATCC 4356, ATCC 15820, 4962, 33200, 4461, *L. reuteri*, *Bifidobacterium longum* 536 and 20099 were obtained from the Victoria University Culture Collection (Werribee, Victoria,

Australia). *B. animalis* Bb12 was obtained from Chr Hansen Pty. Ltd. (Bayswater, Victoria, Australia). The purity of cultures was checked through Gram staining and the organisms were stored at -80°C in 40% glycerol.

3.2.2 Soymilk manufacture

SPI (SUPRO 590) supplied by Solae Co. (Chatswood, N.S.W., Australia) was used in the production of soymilk at 40g per litre of sterilized water according to the method of Tsangalis *et al.* (2002). A 10 L batch of soymilk were manufactured and dispersed into 6 bottles of 1.6 L, then sterilised at 121°C for 15 mins. Each batch was then inoculated with each microorganism.

3.2.3 Fermentation and storage

The remaining 400 mL was aseptically dispersed into 6 equal volumes, inoculated by 6 selected strains and incubated at 37°C for upto 48 h and β -glucosidase activity was determined after 12, 24, 36 and 48 h of incubation. The other batch of 6 bottles containing volumes of 1.6 L each was inoculated (at 5% v/v; 10^7 – 10^8 cfu/g) with each microorganism, incubated for 24 h at 37°C , then aseptically dispersed into 4 sterile clean bottles (400 mL) each and stored at different temperatures (-80°C , 4°C , 25°C and 37°C) for weekly β -glucosidase analysis for up to 8 weeks.

3.2.4 Assay for β -glucosidase activity in Soymilk

Fifteen strains of bacteria were each inoculated in 250 mL of soymilk, and then incubated at 37°C for 48 h and screening for β -glucosidase activity was conducted at 0, 12, 24, 36, and 48 h of incubation. Based on β -glucosidase activity, 6 strains were selected for further enzymatic assay. The strains were activated in MRS broth (De Mann *et al.* 1960) by inoculating 1% level at 37°C for 20 h. The fourth inoculation was done in sterile soymilk from which 5% w/v of each active culture was inoculated in 200 mL of 6 bottles of soymilk. Fifty millilitres of aliquots were

withdrawn aseptically from each sample at 12, 24, 36 and 48 h of incubation and β -glucosidase activity was determined using a modified method of Tsangalis *et al.* (2002) by measuring the rate of hydrolysis of ρ -nitrophenyl β -D-glucopyranoside (ρ NPG). For the storage study, fifty millilitres of aliquots were withdrawn aseptically from each sample at weekly intervals and β -glucosidase activity was determined.

One thousand microlitre of 5mM ρ NPG prepared in 100mM sodium phosphate buffer (pH 7.0) was added to 10 mL of each aliquot and incubated at 37°C for 30 min (Scalabrini *et al.* 1998). Five hundred microlitres of 1M cold sodium carbonate were added to stop the reaction. The aliquots were then placed in 1.8 mL Eppendorf centrifuge tubes followed by centrifugation ($14,000 \times g$ for 30 min) using an Eppendorf centrifuge (model 5415C; Crown Scientific Pty., Ltd., Victoria, Australia). The amount of ρ -nitrophenol released was measured using a spectrophotometer (Pharmacia LKB[®], Novospec II[®], Uppsala, Sweden) at 420 nm. One unit of the enzyme activity was defined as the amount of β -glucosidase that released 1 nanomole of ρ -nitrophenol from the substrate ρ NPG per millilitre per min under assay conditions. The specific activity was expressed as units of enzyme per nanogram of the protein. The protein concentration was determined by a modified version of the Lowry method (Lowry *et al.* 1951) and Rosenberge (1996). The supernatant was filtered through a 0.45 μ m filter membrane in order to filter out ρ -nitrophenol. The ρ NPG substrate and ρ -nitrophenol were purchased from Sigma Chemical Co. (Castle Hill, NSW, Australia).

3.2.5 Experimental design

The β -glucosidase assay during fermentation of soymilk of up to 48 h was determined in duplicate on 3 trials while 8 weeks of storage at different temperatures was performed in

triplicate on 2 trials and the data presented are means of 6 measurements and the results are presented as mean \pm standard error of 6 analyses.

3.2.6 Statistical analysis

To find the difference in β -glucosidase activity in soymilk, means were analysed using one-way analysis of variance (ANOVA) and 95% confidence levels, using Microsoft[®] Excell Statpro[®] as described by Albright *et al.* (1999) for fermentation period. However, β -glucosidase activity during storage at different temperatures was analysed using two-way analysis of variance (ANOVA) and 95% confidence levels. All data with a $P < 0.05$ were classified as statistically significant.

3.3 RESULTS AND DISCUSSION

3.3.1 β -Glucosidase activity the microorganisms

Probiotic microorganisms show varying levels of β -glucosidase, β -galactosidase and α -galactosidase activities (Tochikura *et al.* 1986). According to Tsangalis *et al.* (2003), some strains of *bifidobacterium* showed higher levels of β -galactosidase activity while others such as *B. animalis* Bb12 exhibited lower levels of β -galactosidase but higher levels of β -glucosidase activity. Due to the fact that these three enzymes exist inter-cellularly in crude forms, assaying for a specific enzyme required the use of a specific substrate in order to determine the enzyme potential of a microorganism. Due to our interest in β -glucosidase activity, ρ -nitrophenyl β -D-glucopyranoside (ρ NPG) was used as the substrate. In Table 3.1, the list of 15 strains showing β -glucosidase activity in fermented soymilk after 24 h during a 48 h incubation period at 37°C is shown. Based on β -glucosidase activity in soymilk, it appeared that *L. acidophilus* and *L. casei* strains showed better β -glucosidase activity compared to that of *bifidobacterium*. Strains for

further study were selected based on highest and lowest enzyme activities resulting in a representation of strains from *L. acidophilus*, *L. casei* and *bifidobacterium*.

The β -glucosidase activity of the 6 selected strains over a 48 h incubation period at 37°C is shown in Table 3.2. All microorganisms showed very low β -glucosidase activity under the assay conditions. As a result, the specific activity was expressed as the unit of enzyme activity per nanogram of the protein. There was a significant difference ($P < 0.05$) in the β -glucosidase activity over 12, 24, 36 and 48 h of incubation of *L. acidophilus* 33200, *L. casei* 2607, *L. acidophilus* 4962 and *L. casei* ASCC 290. The enzyme activities of *B. animalis* Bb12 and *L. acidophilus* 4461 did not differ significantly ($P < 0.05$). These differences can be attributed to strain specificity.

In general, the 6 selected strains showed an increase in the β -glucosidase activity over incubation period of up to 24 h followed by a decline as fermentation progressed. The increase in enzyme activity between 12 h to 24 h was significant ($P < 0.05$) for strains of *L. acidophilus* 33200, *L. casei* 2607, *L. acidophilus* 4962 and *L. casei* ASCC 290. *L. acidophilus* 4461 had the highest activity at 24 h, but this was not significantly different from that at 12 h. *B. animalis* Bb12 showed the highest activity after 36 h, but not significantly different ($P < 0.05$) from that at 12 h and at 24 h.

The increase in β -glucosidase activity and the subsequent decline apparently corresponded to the growth of these probiotic microorganisms in the soy media (growth results not shown). As reported by Tsangalis *et al.* (2002), there was a direct correlation between β -glucosidase activity and the growth of *bifidobacterium*. Thus, it appears that, except *B. animalis* Bb12, the strains of *L. acidophilus* and *L. casei* had exponential growth phase between 12 h and 24 h of incubation. *B. animalis* Bb12, which had highest β -glucosidase activity at 36 h, appeared to have much slower growth than the other 5 micro-organisms. These results are in agreement with those of Scalabrini *et al.* (1998) and Tsangalis *et al.* (2002). The growth of probiotic cultures in general is

remarkably slow compared to that of yoghurt starter cultures that take only 4 h to complete the fermentation (Shah, 2000).

Comparatively, *L. acidophilus* 33200 showed the highest β -glucosidase activity (0.635 units of enzyme) among the 5 strains with peak enzyme activity at 24 h, while *B. animalis* Bb12 with peak enzyme activity at 36 h showed least β -glucosidase activity (0.353 units of enzyme). During the period between 12 h and 24 h, the relative increase in β -glucosidase activities among the 6 micro-organisms were highest at 0.294 units for *L. casei* 2607, followed by 0.199 units for *L. casei* ASCC 290, 0.177 units for *L. acidophilus* 33200, 0.137 units for *L. acidophilus* 4962, 0.087 units for *L. acidophilus* 4461 and 0.036 units for *B. animalis* Bb12. *L. casei* strains in general showed greater β -glucosidase activity during the 12 h to 24 h of fermentation period compared to *L. acidophilus* strains, while *bifidobacterium* showed least β -glucosidase activity during the same period.

3.3.2 Stability of β -glucosidase during storage at different temperatures

Tables 3.3 to 3.8 show the β -glucosidase activity in fermented soymilk during 8 week storage at different temperatures for *L. acidophilus* 33200, *B. animalis* Bb12, *L. casei* 2607, *L. acidophilus* 4962, *L. acidophilus* 4461 and *L. casei* ASCC 290, respectively. In general, storage at lower temperatures (-80°C and 4°C) exhibited better β -glucosidase stability as compared to that at higher temperatures (25°C and 37°C). There was no significant difference ($P < 0.05$) in β -glucosidase activity and stability between storage at -80°C and at 4°C. This suggests that low temperatures (refrigerated or frozen) could be used for storage and distribution in the supply chain without any loss of β -glucosidase. The storage temperature appeared to have an important influence on the β -glucosidase activity. According to Bruno & Shah (2003), viability of bifidobacteria cells is determined by the cellular activity and metabolism. Low temperatures restrict cellular activity and metabolism, therefore allowing very small energy losses, thus better

stability of the cells and the enzyme within the cells. The lack of stability at higher storage temperatures could also be as a result of faster change in the pH of the media compared to that at lower storage temperatures (Figure 3.7). The faster change in pH at higher storage temperature is possibly due to the production of lactic and acetic acids.

As shown in the tables, there was in general an accelerated decrease in the β -glucosidase activity from the 6 strains after week 5 of storage at all the temperatures, but especially at higher storage temperatures. It seems probable that viable cells will exhibit enzyme activity, and non-viable cells would not show any enzyme activity, which could as well be an index of cell viability. From the product development perspective, the shelf-life of a functional food product manufactured by use of these probiotic micro-organisms in soymilk would be approximately 5 to 6 weeks.

Table 3.3 shows the β -glucosidase activity of *L. acidophilus* 33200 in soymilk during 8 weeks storage at different temperatures. The enzyme activities were significantly higher ($P < 0.05$) at -80°C and at 4°C of storage as compared to those at 25°C and 37°C after 1 week of storage. In general, the enzyme activity reduced during storage. Although there was a general decrease in the β -glucosidase activity, the values for lower storage temperatures were not significantly ($P < 0.05$) different. Although the values (in units of enzyme) were different for storage at lower temperatures, the differences were not statistically different ($P < 0.05$). The same trend of higher and better stability of β -glucosidase at lower storage temperatures (-80°C and 4°C) compared to higher storage temperatures (25°C and 37°C) was observed for *B. animalis* Bb12 (Table 3.4), *L. casei* 2607 (Table 3.5), *L. acidophilus* 4962 (Table 3.6), *L. acidophilus* 4461 (Table 3.7) and *L. casei* ASCC 290 (Table 3.8). It has been reported that proteins generally show better stability at $\leq 4^{\circ}\text{C}$. Storage at room temperature often leads to protein degradation and/or inactivity (Pierce Technology Inc., 2003). For short-term storage of 1 day to a few weeks, many proteins (including enzymes) may be stored at 4°C but at room temperature or higher, short to long term storage will

lead to loss of stability. Thus the findings in this study suggest that low temperature storage may be suitable for better stability of endogenous β -glucosidase enzyme.

The values of β -glucosidase activity (units of enzymes) during low temperature storage remained generally higher compared to those at higher temperatures throughout the entire storage period. At the end of week 8, the enzyme activity was marginally higher at -80°C compared to that at 4°C for *L. acidophilus* 33200, *L. acidophilus* 4962 and *L. acidophilus* 4461, while marginally higher at 4°C as compared to at -80°C , for *B. animalis* BB12, *L. casei* 2607 and *L. casei* ASCC 290. Although the values were different between storage at 4°C and at -80°C , the differences were not statistically significant ($P < 0.05$).

3.3.3 Stability of β -glucosidase at different storage temperatures; - a critical analysis for 5 weeks

Stability of β -glucosidase activity during the first 5 weeks of storage would be critical as this is the period most likely to be the shelf life of the functional soy based product. Figures 3.1 to 3.6 show the stability of β -glucosidase activity produced by *L. acidophilus* 33200, *B. animalis* Bb12, *L. casei* 2607, *L. acidophilus* 4962, *L. acidophilus* 4461 and *L. casei* ASCC 290, respectively. Stability of β -glucosidase activity could be significant in the stability of soy isoflavone aglycones, which are transformed from their inactive Glycoside forms by these enzymes. The enzyme stability was determined by drawing a regression trendline of β -glucosidase activity during 5 weeks of storage at -80°C , 4°C , 25°C and 37°C .

The trendlines showed a negative slope indicating a general decrease in the β -glucosidase activity during the 5 weeks of storage. The slope magnitude varied with strain and storage temperature. The slope was indicative of the stability of the enzyme; smaller gradient indicated better stability while a bigger one signified less stability. Lower storage temperatures generally showed smaller

gradient/slope in contrast to higher storage temperatures that gave a comparatively larger gradient. Figure 3.1 shows the regression trendlines of β -glucosidase activity of *L. acidophilus* 33200 in soymilk at different temperatures. Storage at -80°C and 4°C had a gradient of 0.0418 and 0.0376 units of enzyme per week, respectively. On the other hand, storage at higher temperatures of 25°C and 37°C had higher slope magnitudes of 0.1509 and 0.0973 units of enzyme per week, respectively, indicating less stability of the enzyme at higher temperatures. The same trend was observed with other strains as shown in Figures 3.2 to 3.6. Amongst the 6 strains, *B. animalis* Bb12 exhibited the best stability of β -glucosidase activity during storage at 4°C , showing a slope of 0.014 units of enzyme per week. The enzyme activity was, however, most unstable during storage at 25°C where the slope magnitude was 0.1546. Thus, stability of endogenous β -glucosidase could be attributed to strain specificity, and is also influenced by storage temperature and the duration of storage.

The equations in the Figures 3.1 to 3.6 also show the y intercept, which is the initial β -glucosidase activity in soymilk. The initial enzyme activity is higher for storage at lower storage temperatures than that at higher storage temperatures. It appears reasonable to assume that 5 weeks of storage as shelf-life for soy based probiotic functional food would be dependent on β -glucosidase activity and the stability of the enzyme. There was no significant difference ($P < 0.05$) in initial β -glucosidase activity for all the 6 micro-organisms during storage at lower temperatures of 4°C and -80°C . The order among the 6 strains for best stability at 4°C was given by *B. animalis* Bb12 with a slope magnitude of 0.014 units enzyme per week, followed by *L. casei* ASCC 290, *L. acidophilus* 4461, *L. acidophilus* 33200, *L. casei* 2607 and *L. acidophilus* 4962 whose regression trendline magnitudes are 0.0249, 0.0284, 0.0376, 0.0396 and 0.0546 units of enzyme per week respectively. Thus, the choice of micro-organism for use in the soy-based functional food will depend on the β -glucosidase activity and its stability at the chosen storage temperature.

3.4 CONCLUSIONS

L. acidophilus 33200, *B. animalis* Bb12, *L. casei* 2607, *L. acidophilus* 4962, *L. acidophilus* 4461 and *L. casei* ASCC 290 produced varying levels of β -glucosidase activity and stability at different storage temperatures. The β -glucosidase activity declined over time. For storage of a product for up to 5 weeks for maintaining appreciable levels of β -glucosidase activity and better stability of the enzyme, storage at 4°C would be ideal as was observed from the slope calculated during storage. Even at the end of 8 weeks storage, it was observed that 3 strains had marginally higher β -glucosidase activity at 4°C compared to at -80°C but the values were non significant ($P < 0.05$). Thus for very long-term storage, a temperature of -80°C may be preferred. β -glucosidase enzyme is important in the biotransformation of the predominant inactive isoflavone Glycosides in soymilk to their bioactive isoflavone aglycone constituents. Thus, the enzyme activity and its stability during storage may influence conversion of inactive isoflavone glycosides to bioactive isoflavone aglycones. The health benefits of bioactive isoflavone aglycones in post-menopausal women have been well documented. A correct storage temperature is therefore important for maintaining high level of β -glucosidase activity from probiotic microorganisms as well as stability of the enzyme in soymilk.

Table 3.1 Peak enzyme activity in fermented soymilk at 24 h of incubation at 37°C

Micro-organisms	Absorbance at 24 h (420nm)	¹ Units of enzyme
<i>L. acidophilus</i> 4461	0.956	2.204
<i>L. casei</i> 2607	0.954	2.199
<i>L. casei</i> ASCC290	0.948	2.184
<i>L. acidophilus</i> ATCC4962	0.932	2.148
<i>L. acidophilus</i> ATCC4356	0.928	2.139
<i>L. casei</i> 1520	0.928	2.139
<i>L. casei</i> ASCC1521	0.923	2.128
<i>L. casei</i> ASCC279	0.921	2.122
<i>L. casei</i> ATCC15286	0.916	2.112
<i>B. animalis</i> Bb12	0.909	2.095
<i>L. casei</i> ASCC292	0.906	2.088
<i>L. acidophilus</i> 33200	0.892	2.056
<i>L. reuteri</i>	0.885	2.039
<i>B. longum</i> 20099	0.867	1.998
<i>B. longum</i> 536	0.855	1.972

¹Mean of units of enzyme (n = 6). One unit of enzyme is the amount of β -glucosidase that released one nanomole of *p*-nitrophenol from *p*NPG per mL/min at 37°C

Table 3.2 β -Glucosidase activity¹ of the selected probiotic micro-organisms in soymilk incubated for 12, 24, 36, and 48 h

Incubation time (h)	<i>L. acidophilus</i> 33200	<i>B. animalis</i> Bb12	<i>L. casei</i> 2607	<i>L. acidophilus</i> 4962	<i>L. acidophilus</i> 4461	<i>L. casei</i> ASCC290
12	0.458 ^a ± 0.019	0.257 ^a ± 0.027	0.315 ^a ± 0.048	0.317 ^a ± 0.047	0.322 ^a ± 0.062	0.338 ^a ± 0.062
24	0.635 ^{acd} ± 0.032	0.293 ^a ± 0.027	0.609 ^b ± 0.061	0.454 ^{ab} ± 0.067	0.409 ^a ± 0.041	0.537 ^b ± 0.028
36	0.312 ^{ab} ± 0.017	0.353 ^a ± 0.034	0.295 ^a ± 0.016	0.277 ^a ± 0.012	0.333 ^a ± 0.051	0.433 ^{ab} ± 0.032
48	0.240 ^b ± 0.078	0.275 ^a ± 0.010	0.229 ^a ± 0.021	0.216 ^a ± 0.051	0.224 ^a ± 0.008	0.323 ^{ab} ± 0.047

Results expressed as means ± standard error of units of enzyme (n = 6).

¹One unit of enzyme is the amount of β -glucosidase that released one nanomole of *p*-nitrophenol from *p*NPG per mL/ min at 37°C

^{a,b,c,d}Means in the same column with different superscripts are significantly different (P < 0.05)

Statistical analysis by means of one-way ANOVA

Table 3.3 β -Glucosidase activity¹ of *Lactobacillus acidophilus* 33200 in soymilk during storage at different temperatures

Storage Temps	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
-80°C	1.371 ^{a,A} ± 0.04	1.243 ^{a,A} ± 0.04	1.255 ^{a,A} ± 0.05	1.187 ^{a,A} ± 0.04	1.190 ^{a,A} ± 0.04	0.922 ^{b,A} ± 0.03	0.444 ^{bc,A} ± 0.03	0.571 ^{bc,A} ± 0.03
4°C	1.225 ^{a,A} ± 0.05	1.188 ^{a,A} ± 0.03	1.187 ^{a,A} ± 0.03	1.082 ^{a,A} ± 0.02	1.090 ^{a,A} ± 0.02	0.802 ^{b,A} ± 0.01	0.546 ^{bc,A} ± 0.02	0.526 ^{bc,A} ± 0.02
25°C	0.880 ^{a,B} ± 0.03	0.871 ^{a,B} ± 0.03	0.850 ^{a,B} ± 0.03	0.432 ^{bc,B} ± 0.02	0.345 ^{bd,B} ± 0.01	0.345 ^{bd,B} ± 0.01	0.139 ^{bcd,C} ± 0.01	0.145 ^{bcd,B} ± 0.01
37°C	0.730 ^{a,B} ± 0.03	0.682 ^{a,C} ± 0.02	0.670 ^{a,B} ± 0.03	0.461 ^{b,B} ± 0.02	0.355 ^{bc,B} ± 0.02	0.295 ^{bc,B} ± 0.01	0.265 ^{bc,B} ± 0.01	0.075 ^{bc,C} ± 0.02

Results expressed as mean ± standard error of units of enzyme (n = 6). Statistical analysis by means of two-way ANOVA

¹One unit of enzyme is the amount of β -glucosidase that released one nanomole of *p*-nitrophenol from *p*NPG per mL/ min at 37°C

^{a,b,c,d,e} Means in the same row with different lowercase superscripts are significantly different (P < 0.05)

^{A,B,C,D} Means in the same column with different uppercase superscripts are significantly different (P < 0.05).

Table 3.4 β -Glucosidase activity¹ of *Bifidobacterium animalis* Bb12 in soymilk during storage at different temperatures

Storage Temps	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
-80°C	1.076 ^{a,A} ± 0.02	1.048 ^{a,A} ± 0.02	1.014 ^{ac,A} ± 0.02	0.970 ^{ad,AB} ± 0.02	0.927 ^{bcd,AB} ± 0.04	0.692 ^{b,AB} ± 0.01	0.580 ^{b,B} ± 0.01	0.614 ^{bc,A} ± 0.01
4°C	1.251 ^{a,A} ± 0.02	1.203 ^{a,A} ± 0.02	1.224 ^{a,A} ± 0.02	1.229 ^{a,A} ± 0.01	1.168 ^{a,A} ± 0.02	0.831 ^{bc,A} ± 0.01	0.894 ^{bd,A} ± 0.01	0.652 ^{b,A} ± 0.01
25°C	1.447 ^{a,A} ± 0.02	1.170 ^{bcd,A} ± 0.02	0.992 ^{bc,B} ± 0.03	0.973 ^{bc,AB} ± 0.03	0.797 ^{ac,AB} ± 0.05	0.645 ^{bdef,AB} ± 0.01	0.312 ^{bdeg,C} ± 0.01	0.498 ^{bdef,B} ± 0.01
37°C	0.366 ^{a,B} ± 0.06	0.167 ^{bcd,B} ± 0.03	0.102 ^{bc,C} ± 0.02	0.095 ^{bc,C} ± 0.02	0.087 ^{bc,C} ± 0.02	0.044 ^{bc,C} ± 0.01	0.086 ^{bc,D} ± 0.01	0.013 ^{bd,C} ± 0.01

Results expressed as mean ± standard error of units of enzyme (n = 6). Statistical analysis by means of two-way ANOVA

¹One unit of enzyme is the amount of β -glucosidase that released one nanomole of *p*-nitrophenol from *p*NPG per mL/ min at 37°C

^{a,b,c,d,e} Means in the same row with different lowercase superscripts are significantly different (P < 0.05)

^{A,B,C,D} Means in the same column with different uppercase superscripts are significantly different (P < 0.05).

Table 3.5 β -Glucosidase activity¹ of *Lactobacillus casei* 2607 in soymilk during storage at different temperatures

Storage Temps	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
-80°C	1.232 ^{a,A} ± 0.02	1.189 ^{a,A} ± 0.02	1.158 ^{a,A} ± 0.01	1.016 ^{b,A} ± 0.01	1.065 ^{b,A} ± 0.01	0.826 ^{bc,A} ± 0.01	0.392 ^{bcd,A} ± 0.01	0.335 ^{bcd,A} ± 0.01
4°C	1.174 ^{a,A} ± 0.02	1.104 ^{ac,A} ± 0.01	1.084 ^{ad,A} ± 0.02	1.012 ^{bcd,A} ± 0.01	1.021 ^{bcd,A} ± 0.02	0.793 ^{b,A} ± 0.01	0.306 ^{be,A} ± 0.01	0.346 ^{bef,A} ± 0.01
25°C	1.117 ^{a,A} ± 0.02	1.084 ^{a,A} ± 0.03	0.961 ^{a,AB} ± 0.01	0.927 ^{a,AB} ± 0.02	0.882 ^{b,B} ± 0.03	0.827 ^{b,A} ± 0.02	0.111 ^{bcd,BC} ± 0.01	0.068 ^{bcd,B} ± 0.01
37°C	0.640 ^{a,B} ± 0.02	0.632 ^{a,B} ± 0.01	0.426 ^{b,C} ± 0.01	0.511 ^{b,C} ± 0.02	0.411 ^{bde,C} ± 0.03	0.347 ^{be,B} ± 0.01	0.153 ^{bef,B} ± 0.01	0.042 ^{bc,B} ± 0.01

Results expressed as mean ± standard error of units of enzyme (n = 6). Statistical analysis by means of two-way ANOVA

¹One unit of enzyme is the amount of β -glucosidase that released one nanomole of p -nitrophenol from p NPG per mL/ min at 37°C

^{a,b,c,d,e} Means in the same row with different lowercase superscripts are significantly different (P < 0.05)

^{A,B,C,D} Means in the same column with different uppercase superscripts are significantly different (P < 0.05).

Table 3.6 β -Glucosidase activity¹ of *Lactobacillus acidophilus* 4962 in soymilk during storage at different temperatures

Storage Temps	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
-80°C	1.236 ^{a,A} ± 0.01	1.192 ^{a,A} ± 0.01	1.153 ^{ab,A} ± 0.01	1.112 ^{ab,A} ± 0.01	1.079 ^{b,A} ± 0.01	0.869 ^{bc,A} ± 0.01	0.444 ^{bcd,A} ± 0.01	0.341 ^{bcd,e,A} ± 0.02
4°C	1.258 ^{a,A} ± 0.03	1.127 ^{b,A} ± 0.01	1.058 ^{b,A} ± 0.02	1.106 ^{b,A} ± 0.03	1.019 ^{b,A} ± 0.02	0.887 ^{bc,A} ± 0.01	0.455 ^{bcd,A} ± 0.01	0.308 ^{bcd,e,A} ± 0.01
25°C	0.862 ^{a,B} ± 0.01	0.789 ^{b,B} ± 0.01	0.701 ^{bc,B} ± 0.01	0.633 ^{cd,B} ± 0.02	0.615 ^{cd,B} ± 0.01	0.611 ^{bc,B} ± 0.01	0.121 ^{bef,B} ± 0.01	0.276 ^{befg,B} ± 0.01
37°C	0.607 ^{a,C} ± 0.01	0.567 ^{a,C} ± 0.02	0.382 ^{b,C} ± 0.01	0.270 ^{bc,C} ± 0.01	0.253 ^{bc,C} ± 0.02	0.261 ^{bc,C} ± 0.01	0.075 ^{bcd,C} ± 0.01	0.040 ^{bc,C} ± 0.01

Results expressed as mean ± standard error of units of enzyme (n = 6). Statistical analysis by means of two-way ANOVA

¹One unit of enzyme is the amount of β -glucosidase that released one nanomole of *p*-nitrophenol from *p*NPG per mL/ min at 37°C

^{a,b,c,d,e} Means in the same row with different lowercase superscripts are significantly different (P < 0.05)

^{A,B,C,D} Means in the same column with different uppercase superscripts are significantly different (P < 0.05).

Table 3.7 β -glucosidase activity¹ of *Lactobacillus acidophilus* 4461 in soymilk during storage at different temperatures

Storage Temps	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
-80°C	1.269 ^{a,A} ± 0.01	1.159 ^{b,A} ± 0.01	1.080 ^{bc,A} ± 0.01	1.147 ^{b,A} ± 0.01	1.121 ^{b,A} ± 0.01	1.008 ^{bcd,A} ± 0.01	0.722 ^{bcde,A} ± 0.01	0.602 ^{bcde,A} ± 0.01
4°C	1.420 ^{a,A} ± 0.04	1.335 ^{abc,A} ± 0.02	1.291 ^{bc,A} ± 0.02	1.301 ^{bc,A} ± 0.04	1.294 ^{bc,A} ± 0.04	1.021 ^{b,A} ± 0.02	0.502 ^{bd,A} ± 0.02	0.392 ^{bd,A} ± 0.01
25°C	0.538 ^{a,B} ± 0.01	0.451 ^{ab,B} ± 0.02	0.441 ^{ab,B} ± 0.02	0.404 ^{bc,B} ± 0.01	0.370 ^{cd,B} ± 0.01	0.254 ^{cde,B} ± 0.02	0.179 ^{cdf,B} ± 0.01	0.061 ^{def,B} ± 0.03
37°C	0.253 ^{a,C} ± 0.07	0.184 ^{ab,C} ± 0.09	0.015 ^{ad,C} ± 0.01	0.011 ^{bcd,C} ± 0.01	0.008 ^{bcd,C} ± 0.01	0.008 ^{bcd,C} ± 0.01	0.006 ^{bce,C} ± 0.01	0.006 ^{bce,C} ± 0.01

Results expressed as mean ± standard error of units of enzyme (n = 6). Statistical analysis by means of two-way ANOVA

¹One unit of enzyme is the amount of β -glucosidase that released one nanomole of *p*-nitrophenol from *p*NPG per mL/ min at 37°C

^{a,b,c,d,e} Means in the same row with different lowercase superscripts are significantly different (P < 0.05)

^{A,B,C,D} Means in the same column with different uppercase superscripts are significantly different (P < 0.05).

Table 3.8 β -glucosidase activity¹ of *Lactobacillus casei* ASCC 290 in soymilk during storage at different temperatures

Storage Temps	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
-80°C	1.345 ^{a,A} ± 0.02	1.342 ^{a,A} ± 0.01	1.279 ^{ab,A} ± 0.01	1.271 ^{ab,A} ± 0.01	1.243 ^{bc,A} ± 0.01	1.125 ^{bc,A} ± 0.01	0.642 ^{cde,AB} ± 0.01	0.540 ^{de,AB} ± 0.01
4°C	1.175 ^{a,A} ± 0.02	1.152 ^{ab,A} ± 0.02	1.127 ^{ab,A} ± 0.02	1.101 ^{acd,A} ± 0.01	1.076 ^{bcd,A} ± 0.02	0.852 ^{b,B} ± 0.01	0.823 ^{b,A} ± 0.01	0.739 ^{be,A} ± 0.01
25°C	0.629 ^{a,B} ± 0.03	0.623 ^{a,B} ± 0.02	0.618 ^{a,B} ± 0.01	0.507 ^{ab,B} ± 0.03	0.469 ^{bc,B} ± 0.03	0.386 ^{cd,C} ± 0.01	0.311 ^{cde,C} ± 0.02	0.232 ^{cd,C} ± 0.01
37°C	0.546 ^{a,B} ± 0.02	0.477 ^{ab,BC} ± 0.01	0.343 ^{b,C} ± 0.01	0.255 ^{bc,C} ± 0.03	0.202 ^{bcd,C} ± 0.03	0.133 ^{bce,D} ± 0.01	0.118 ^{cde,D} ± 0.03	0.067 ^{def,D} ± 0.02

Results expressed as mean ± standard error of units of enzyme (n = 6). Statistical analysis by means of two-way ANOVA

¹One unit of enzyme is the amount of β -glucosidase that released one nanomole of *p*-nitrophenol from *p*NPG per mL/ min at 37°C

^{a,b,c,d,e} Means in the same row with different lowercase superscripts are significantly different (P < 0.05)

^{A,B,C,D} Means in the same column with different uppercase superscripts are significantly different (P < 0.05)

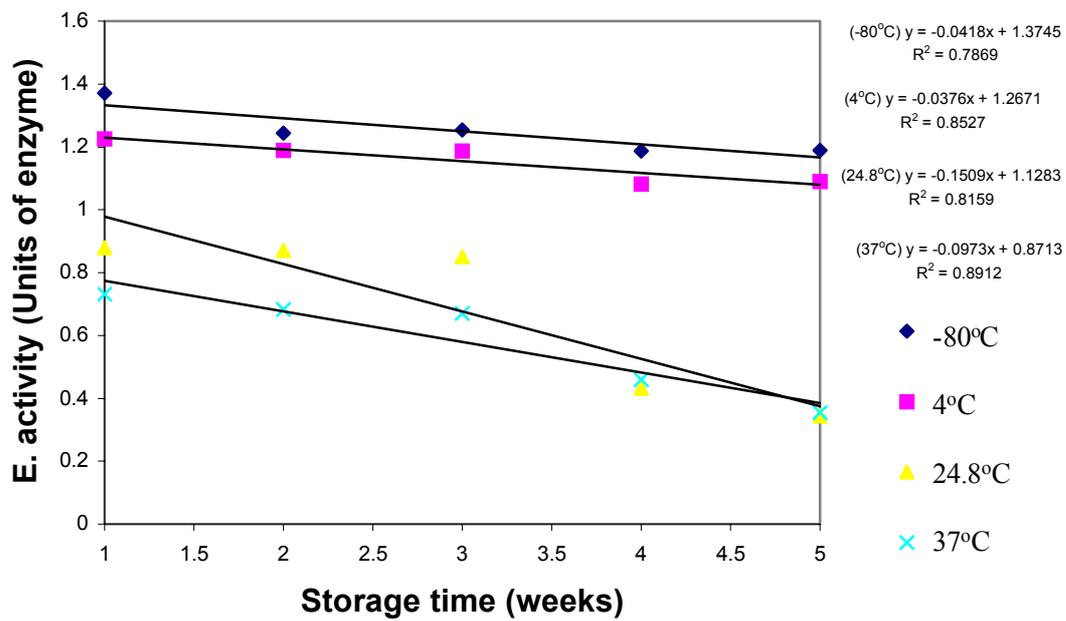


Figure 3.1 β -Glucosidase activity in soymilk by *L. acidophilus* 33200 during storage at different temperatures

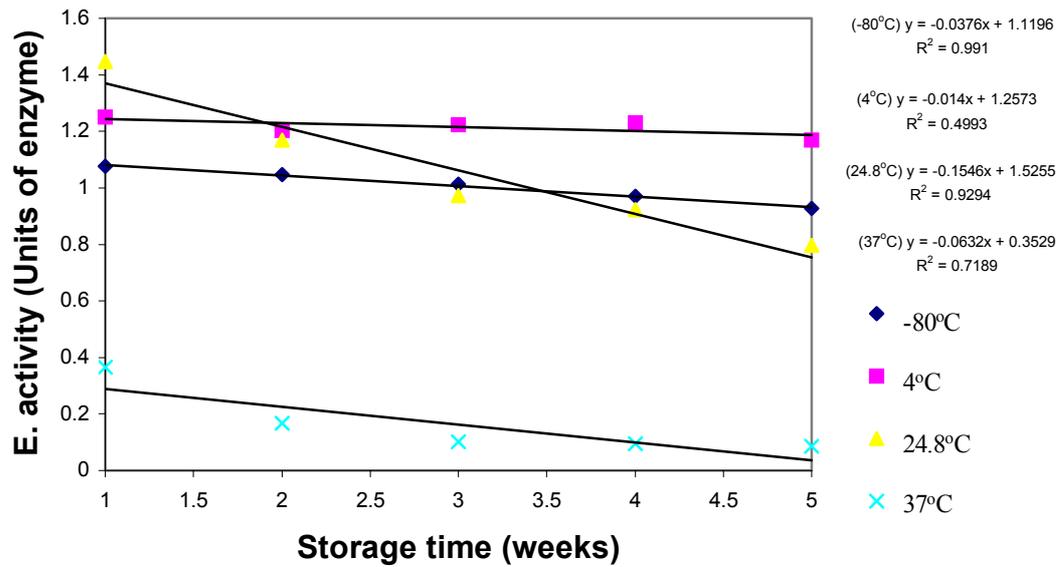


Figure 3.2 β -Glucosidase activity in soymilk by *B. animalis* Bb12 during storage at different temperatures

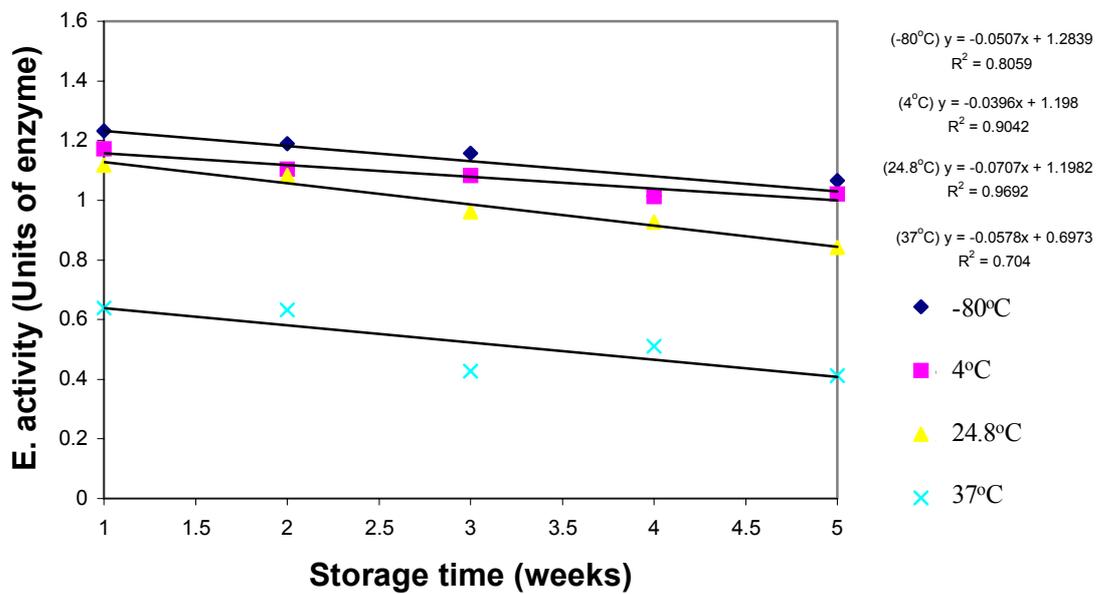


Figure 3.3 β -Glucosidase activity in soymilk by *L. casei* 2607 during storage at different temperatures

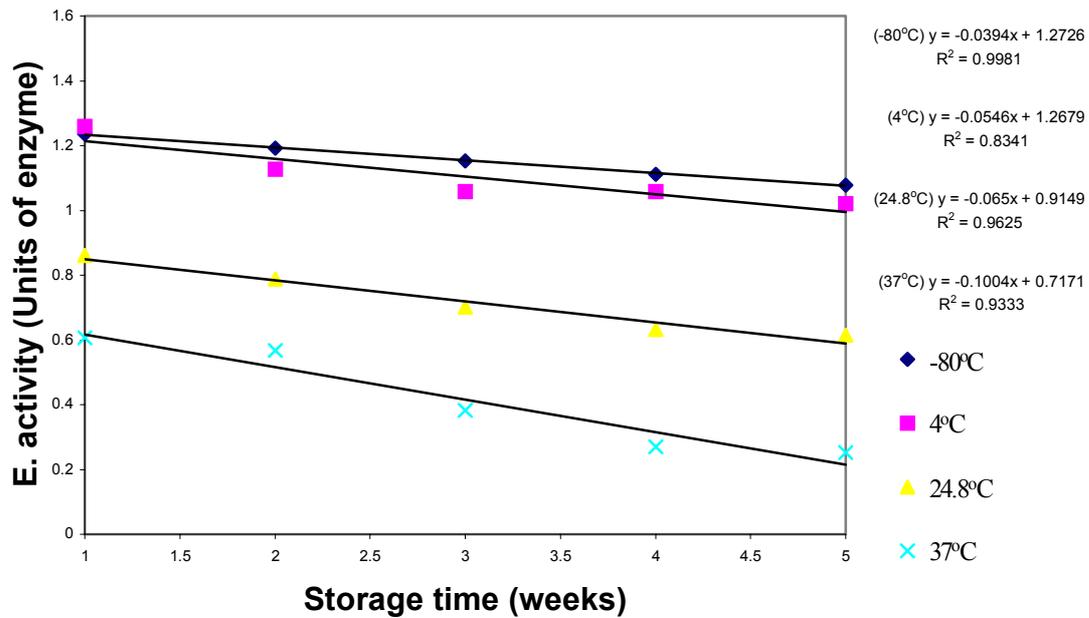


Figure 3.4 β -Glucosidase activity in soymilk by *L. acidophilus* 4962 during storage at different temperatures

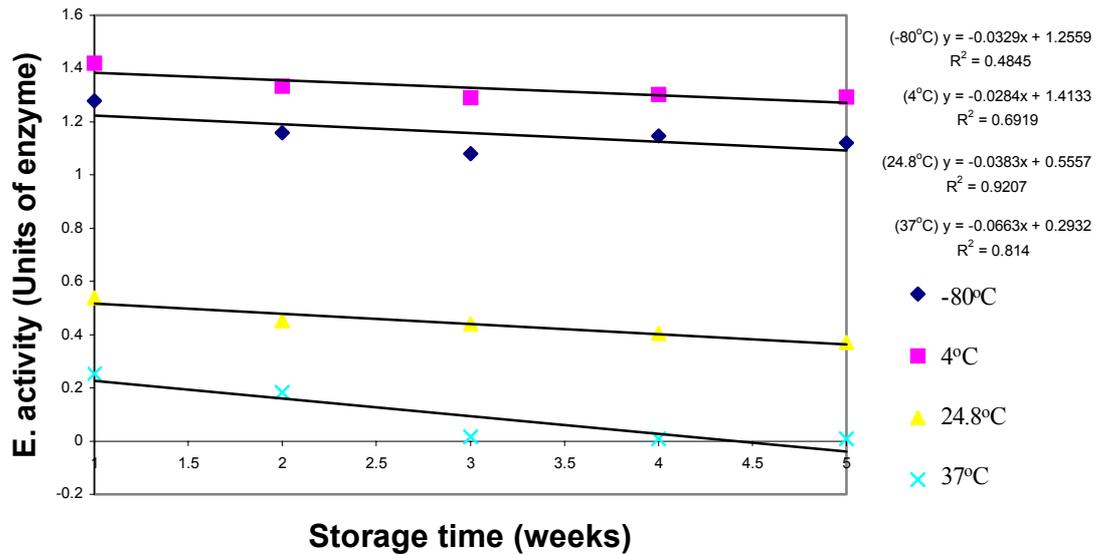


Figure 3.5 β -Glucosidase activity in soymilk by *L. acidophilus* 4461 during storage at different temperatures

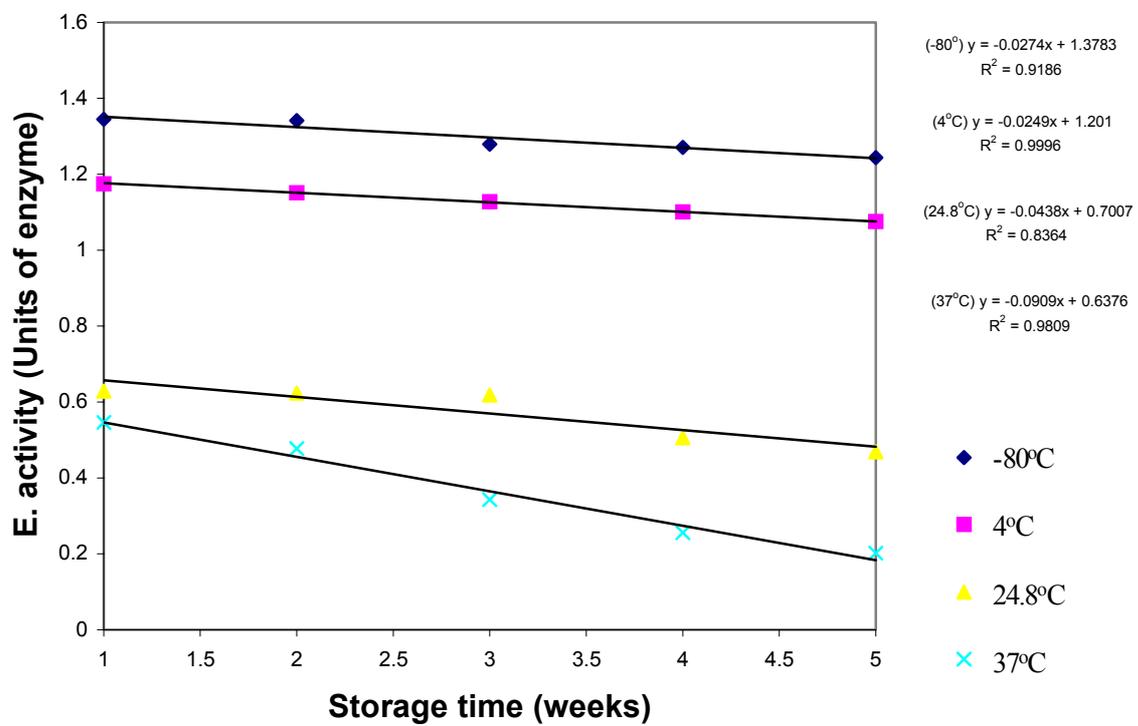


Figure 3.6 β -Glucosidase activity in soymilk by *L. casei* ASCC 290 during storage at different temperatures

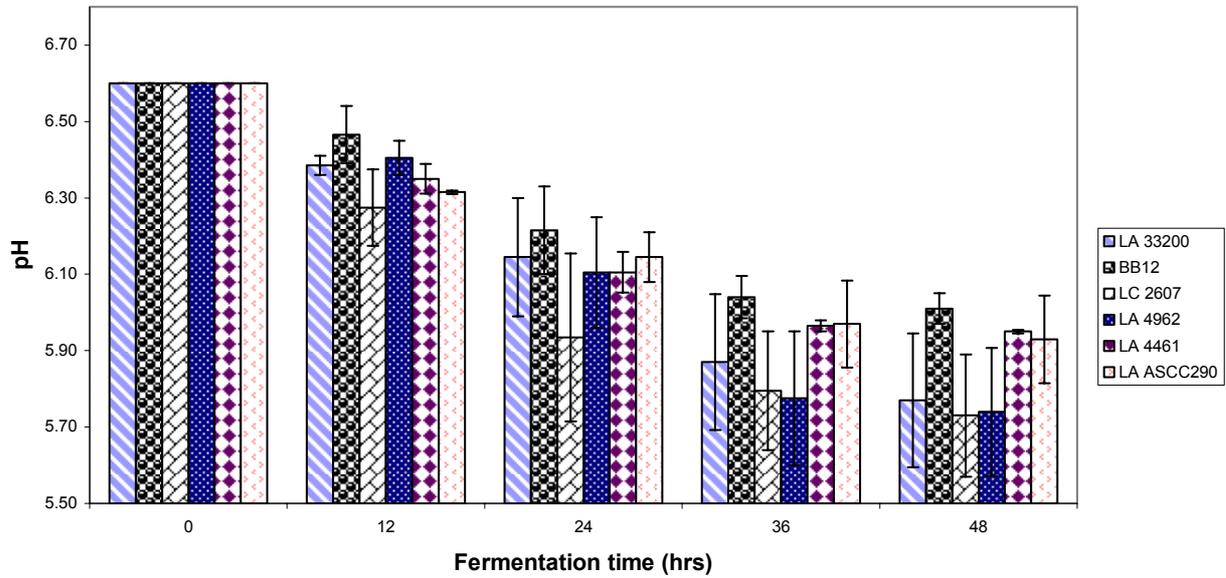


Figure 3.7 pH changes in soymilk during fermentation with each of the 6 microorganisms at 37°C

4.0 Evaluation of Enzymic Potential for Biotransformation of Isoflavone Phytoestrogen in Soymilk by *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus casei*

4.1 INTRODUCTION

Studies investigating the metabolic properties of isoflavones have indicated that they are readily absorbed, metabolised, and excreted, although individual and sex-related differences have been reported (Munro *et al.* 2003). The phytoestrogens found abundantly in soybeans consist of the diphenolic, isomeric family of compounds known as isoflavones. There are three structural “families” of the isoflavones found in soy foods namely, the aglycones, glycosides, malonyl-, and acetyl-glycosides (King & Bignell, 2000). Isoflavones, have been consumed in substantial quantities by those populations whose soy intake is high. Asian populations, with their high intake (50 to 70 mg/d) of soy-derived isoflavones, are known to have a low incidence of osteoporosis, menopausal symptoms and mortality from cardiovascular disease (Nagata *et al.* 1998). On the other hand, isoflavone intake is generally less than 2 mg per day in Western countries (de Kleijn *et al.* 2001).

Generally, the processing of soybeans for the manufacture of soy containing food products increases the hydrolysis of isoflavone glycosides, resulting in higher concentrations of aglycones (Hutchins *et al.* 1995). The biological activity and metabolic fate of dietary soy isoflavones differ depending on their chemical forms (Cassidy, 1996). Since the structure itself is a limiting factor for absorption from gastrointestinal tract (Hendrich *et al.* 1999), the chemical forms of the isoflavone and their metabolites influence the extent of absorption, with aglycones more readily absorbed and more bioavailable than highly polar conjugated species (Setchell, 2000).

The bioavailability of isoflavones (like most nutraceuticals) is usually evaluated in terms of plasma concentrations and / or urinary excretion. In terms of absorption into the plasma and urinary excretion, isoflavone glycosides are not absorbed intact through the intestinal epithelium, and glucuronides are the principal form found in plasma. That is, the glycoside forms must be first hydrolysed by β -glucosidases of gut microflora to be absorbed *in vivo* (Hendrich & Murphy, 2001; Setchell *et al.* 2002b). This knowledge (and some *in vivo* results showing superior estrogenic effects of genistein over its glycosides) has led to the development of aglycon-enriched products, directly by β -glucosidase treatment (Park *et al.* 2002, 2003) or by fermentation with bifidobacteria (Tsangalis *et al.* 2003).

Bifidobacterium and *Lactobacillus* are the predominant members of the intestinal microflora and being classified as probiotics, which are defined as live microbial feed supplement that provides beneficial effects on the host. Probiotics can be used improve the biological activity of soymilk due to the occurrence of endogenous β -glucosidase enzymes within the bacterial cell (Chapter 3.0). The physiological function of the viable cells can be best determined by analysing the enzymic functions of these cells (Chapter 3.0). However, due to the varying β -glucosidase activity in soymilk from these microorganisms, it is important to evaluate this enzymic potential for the possible screening of microorganisms that would be of use to increase the bioactive isoflavone aglycone concentration in fermented soymilk.

The aims of this study were to evaluate the hydrolytic potential of β -glucosidase of *Bifidobacterium*, *L. acidophilus* and *L. casei* for biotransformation of isoflavone glycosides in soymilk and to establish a set of criteria for selection of probiotic micro-organisms based on biotransformation of isoflavones in soymilk during incubation at 37°C.

4.2 MATERIALS AND METHODS

4.2.1 Bacteria

Pure cultures of *L. casei* ASCC 290, *L. casei* 2607, *L. acidophilus* 4962, *L. acidophilus* 33200, and *L. acidophilus* 4461 were obtained from the culture collection mentioned in section 3.2.1. The purity of cultures too was confirmed according to the procedure outlined in section 3.2.1.

4.2.2 Bacterial growth media

Rehydrated de Mann Rogosa Sharpe (MRS) broth (de Mann *et al.* 1960), pH adjusted to 6.7 using 5 M sodium hydroxide, was prepared according to manufacturer instructions (Oxoid Ltd., West Heidelberg, Vic., Australia) and autoclaved at 121°C for 15 min.

4.2.3 Soymilk manufacture

Soy protein isolate (SPI; SUPRO 590), supplied by Solae Co. (Chatswood, N.S.W., Australia), was manufactured as mentioned in section 3.2.2. After reconstitution, the soymilk was dispensed in six 250 mL glass bottles and autoclaved at 121°C for 15 min. After cooling to room temperature, the pH was adjusted in the laminar flow to 6.7 using 5 M sodium hydroxide.

4.2.4 Assay for β -glucosidase activity in soymilk

The 6 microorganisms were individually inoculated in soymilk and β -glucosidase activity was determined at 12, 24, 36 and 48 h of incubation. The activation in MRS and incubation was carried out according to section 3.2.4. Subsequently, 10 mL of active culture was inoculated in triplicate into 250 mL of each of the batches of soymilk (5% w/v) and incubated at 37°C for 48 h. Fifty millilitre aliquots were withdrawn aseptically from each sample at 12, 24, 36, and 48 h of

incubation and the enzyme activity was determined immediately. The rest of the enzyme assay was also conducted according to the process outlined in section 3.2.4.

4.2.5 Fermentation of soymilk with probiotics

For soymilk fermentation studies, 250 mL of sterile soymilk (in glass bottles) was inoculated (in triplicate) with active culture of *Bifidobacterium*, *L. acidophilus* and *L. casei* strains using 5% inoculum and incubated at 37°C for 48 h. Aliquots of 50 mL were withdrawn aseptically at 0, 12, 24, 36, and 48 h of incubation of which 20 mL was for β -glucosidase activity while the remaining 30 mL was stored immediately at -80°C for isoflavone analysis. Measurement of pH was also taken at 12, 24, 36, and 48 h of incubation. The frozen 30 mL aliquot was freeze-dried using a Dynavac® FD300 freeze drier (Rowville, Vic., Australia) for isoflavone extraction and analysis using high-performance liquid chromatography (HPLC).

4.2.6 Extraction of isoflavones for HPLC analysis

The extraction of isoflavones, including malonyl-, acetyl-, β -glycosides, and aglycones from fermented and non-fermented soymilk was performed in triplicate, using a modified version of the method described by Tsangalis *et al.* (2002). A 1-g freeze-dried sample was added to 50 mL of methanol in a 150 mL round bottom flask and refluxed on a heating mantle for 1 h. The mixture was then filtered through a Whatman No. 1 filter paper into a 100 mL volumetric flask. The remaining dried soy matter was washed with the filtered portion and then refiltered into the same flask. A 5 mL aliquot was mixed with 60 μ L of internal standard (ISTD) flavone solution (10 mg/50 mL) and dried under a stream of nitrogen using a Techne Sample Concentrator (Pearce Biotechnology Inc., Rockford, IL., USA.). The resultant dried matter was then resuspended in 1 mL of 10 mM ammonium acetate buffer (containing 0.1% trifluoro-acetic acid) and acetonitrile

(50:50) solution and centrifuged ($14,000 \times g$) for 30 min using an Eppendorf centrifuge (model 5415C; Crown Scientific Pty. Ltd., Vic., Australia) to precipitate undissolved matter prior to transferring to HPLC vials.

4.2.7 Isoflavone standards

All the aglycone standards of genistein, daidzein and glycitein as well as flavone (ISTD) were purchased from Sigma (Castle Hill, N.S.W., Australia) while the β -glycoside standards of genistin, daidzin and glycitin as well as daidzein metabolite equol were purchased from Indofine Chemical Co. (Sommerville, NJ, USA). Genistein, genistin, flavone, daidzein, and equol were prepared in HPLC grade methanol, and daidzin, glycitein and glycitin in ethanol due to their varied solubility characteristics.

4.2.8 Reversed-phase HPLC apparatus and reagents

Isoflavone analyses were carried out on a Hewlett Packard® 1100 series HPLC (Agilent Technologies, Forest Hill, Vic., Australia) with autosampler, quaternary pump, diode array ultraviolet (UV) visible detector, vacuum degasser, and thermostatically controlled column compartment. A keystone Scientific® (Bellefonte, PA, USA) ODS-C18 (250 mm \times 4.6 mm internal diameter, 5 μ m) reversed-phase column was used to separate the isoflavone isomers. HPLC-grade methanol and acetonitrile were purchased from Labscan Analytical Sciences (Bangkok, Thailand), and trifluoro-acetic acid, absolute ethanol, and ammonium acetate from Sigma. All reagents used in isoflavone extraction and HPLC analyses were filtered through a 0.5- μ m FH membrane (Millipore®, Bedford, MA, USA).

4.2.9 HPLC analysis of isoflavones

HPLC gradient elution for isolating the isoflavones for detection was acetonitrile (Solvent A) and 10 mM ammonium acetate buffer containing 0.1% trifluoro-acetic acid (Solvent B) set at a flow rate of 1 mL/min (Setchell *et al.* 2001). After the 20 µl injection of sample or isoflavone standard onto the column, solvent B was set at 100% for 2 min, reduced to 60% over 22 min and finally 100% for 5 min prior to the next injection. A diode array UV-visible detector was set at dual wavelengths of 260 nm to detect the malonyl-, acetyl-, and β-Glycosides, aglycones, and the flavone (ISTD), and 280 nm to detect equol. Single standards were prepared for identification of peak. Malonyl- and acetyl-glycoside conjugates were quantified with respect to their β-glycoside equivalent response factors and corrected according to molecular weight. Isoflavone concentrations were calculated back to wet basis (mg isoflavones/ 100 mL soymilk).

4.2.10 Statistical analysis

The enzyme activity in soymilk and isoflavone concentrations during incubation were obtained in triplicate on two occasions and presented as means ± standard error of 6 replicates. The analysis was conducted using one-way analysis of variance (ANOVA) and 95% confidence levels, using Microsoft[®] Excel Statpro[®] as described by Albright *et al.* (1999). ANOVA data with a P < 0.05 was classified as statistically significant.

4.3 RESULTS AND DISCUSSION

4.3.1 β-Glucosidase activity in soymilk

The result of the enzyme activity in soymilk, discussed in section 3.3.1 is shown as Table 3.2.

Tochikura *et al.* (1986) found that β-glucosidase activity of 12 strains of *Bifidobacterium* sp. propagated in glucose-supplemented broth varied significantly with 2 strains showing no β-

glucosidase activity even after 12 h of incubation. Tsangalis *et al.* (2002) also showed that 4 of 5 strains showed detectable levels of β -glucosidase activity in nutrient media. In hydrolysing the glycosidic bond, the isoflavone glycosides are broken down to their bioactive aglycone forms. Interestingly, the bioconversion of isoflavone glycosides into the bioactive aglycone forms as shown in Tables 4.1 – 4.6 followed the same pattern of β -glucosidase activity (Table 3.2) during the incubation of all the 6 micro-organisms. It was interesting to note that any increase or decrease in β -glucosidase activity was largely corresponding to the appearance of isoflavone aglycone forms.

4.3.2 HPLC analysis of isoflavones in soymilk

Soymilk made from soy protein isolate SPI SUPRO 590 had a total isoflavone concentration of 4.140 mg per 100 mL of soymilk. Figure 4.1 is a chromatogram showing peaks of isoflavone compounds indicating the relative composition of isoflavone constituents in the unfermented soymilk (0 h). The total isoflavone glycosides, which include the β -glycosides, the malonyl and the acetyl forms, were about 90% while the total aglycones constituted only about 10%. This composition of isoflavone glycosides and aglycones falls within the range of these isoflavones in unfermented soy-based foods reported in previous studies. King and Bignell (2000) and Murphy *et al.* (1999) report a range of 80 – 95% of total isoflavone glycosides and 7 – 15% of bioactive aglycones present in the unfermented soy foods. The biologically active estrogen-like isomers are the aglycone forms of genistein, daidzein and glycitein (Setchell & Cassidy, 1999). The aglycone isomers have a structural similarity to human estrogen (estradiol), hence have the capacity to mimic the function of estradiol in the human body (Setchell *et al.* 1999). The importance of our study lies in the results showing that fermentation causes the majority of the isoflavone

glycosides to be transformed into bioactive aglycone forms via β -glucosidase induced hydrolysis, thus shifting the balance of aglycone concentration for the potential health benefit of consumers.

The approximate retention times of malonyl-, acetyl-, β -glycosides and aglycone isoflavone isomers found in soymilk are also shown in Figure 4.1. Malonylglycoside isoflavones eluted first, followed in order by β -glycosides, acetylglycosides and finally aglycones. The isoflavone isomers eluted according to their polarity and hydrophobic interactions with the reversed-phase HPLC column. Thus malonylglycosides with the highest polarity, and having the least affinity to the column surface eluted first followed by less polar compounds with respect to their chemical structures and number of hydroxyl groups. This order of elution was slightly different from that obtained by King & Bignell (2000) where isoflavone β -glycosides eluted first followed in order by malonylglycosides, acetylglycosides and aglycones when using a complex gradient elution system of 0.1% trifluoroacetic acid and acetonitrile as mobile phases. In our study, within each chemical form, daidzein consistently eluted first, followed by glycitein and genistein (Figure 4.1), in agreement with King & Bignell (2000) and Coward *et al.* (1998). The internal standard used in our study was flavone, which eluted at 28.8 min and was well separated from other isomers to prevent co-elution (Figure 4.1). All 12 isoflavone compounds were separated using a gradient elution and detected at 260 nm, near each of their UV absorption maxima. However, equol standard was detected at the UV absorption maximum of 280 nm and eluted very closely with genistein at 22 min even though genistein was better detected at 260 nm. A new method had to be designed to achieve a better separation of genistein and equol in standard solutions as well as those occurring in samples. In general, equol was difficult to detect using a diode array detector in the ultra-violet range and more so if the concentration was low. According to Tsangalis *et al.* (2002), an equol concentration of ≤ 20 ng per 10 μ l injection volume would not have been detectable in the UV range. Similarly, Frank *et al.* (1998) found difficulty in detecting equol in

urine using a diode array detector, with a detection limit that was 3 and 8 times higher than that of daidzein and genistein, respectively. It has been suggested that the difficulty in detecting equol may be caused by the reduction of daidzein to equol (Ollis, 1962), which involves the removal of a carbonyl group and double bond from the pran ring of daidzein.

The peaks of isoflavones isomers found in unfermented soymilk (0 h) are shown in Figure 4.1. β -glycoside forms of isoflavones such as daidzin, glycitin and genistin of unfermented soymilk contributed the highest concentration of (83%) of the total, with a total of 3.262 mg isoflavones per 100 mL at 0 h (Tables 4.1 –4.6). The concentration of β -glycoside genistin was also the highest of the individual isomers, at 2.199 mg isoflavones per 100 mL (Tables 4.1 – 4.6) and also showed the highest peak (f; Figure 1). On the other hand, the concentration of bioactive isoflavone aglycones before fermentation was very low (10% of the total isoflavone content) (Tables 4.1 – 4.6). Acetylglycosides and malonylglycosides, other biologically inactive β -Glycosides forms were found at 4% and 3% of the total isoflavone content corresponding to 0.196 and 0.111 mg per 100 mL of soymilk, respectively (Tables 4.1 – 4.6). Similar results have also been obtained in previous studies by King and Bignell (2000) who found that β -Glycoside forms comprised greater than 80% of the total isoflavone concentration of soymilk followed by aglycone and malonyl and acetylglycoside forms. Equol was not detected in the non-fermented soymilk (Figure 4.1). This was expected, as it is a product of microbial induced reduction of daidzein (Tsangalis *et al.* 2002).

4.3.3 Biotransformation of isoflavone compounds in fermented soymilk

During fermentation of soymilk with probiotic microorganisms exhibiting β -glucosidase activity, a concomitant enzymatic hydrolysis of isoflavone glycosides occurs, leading to changes in the concentration of all the isoflavone forms in soymilk. As shown in Tables 4.1 to 4.6, there were

changes in isoflavone concentration in soymilk during 48 h fermentation with *L. acidophilus* 33200, *B. animalis* Bb12, *L. casei* 2607, *L. acidophilus* 4962, *L. acidophilus* 4461 and *L. casei* ASCC 290, respectively. All the 6 microorganisms caused a significant increase ($P < 0.05$) in the concentration of isoflavone aglycones via the β -glucosidase catalysed hydrolysis of isoflavone glycoside conjugates. At the same time, the concentrations of the biologically inactive isoflavone β -glycosides significantly reduced ($P < 0.05$). Malonyl and acetylglycosides in general did not undergo significant changes in concentration during the fermentation but were also hydrolysed during fermentation even though they remained relatively low in concentration. The concentration of individual aglycone, including daidzein, glycitein and genistein increased significantly ($P < 0.05$) as early as 12 h for *L. acidophilus* 33200, *L. acidophilus* 4962, *L. acidophilus* 4461 and *L. casei* ASCC 290 while it took 24 h for a significant increase ($P < 0.05$) for *B. animalis* Bb12 and *L. casei* 2607. Interestingly, the increase in total aglycone concentrations (Tables 4.1 –4.6) followed the same pattern of β -glucosidase activity (Table 3.2) and peak β -glucosidase activity corresponded with peak aglycone concentrations signifying the hydrolytic function of the enzyme for transformation of isoflavone. Peak isoflavone aglycone concentrations occurred at 12 h during fermentation by *L. casei* ASCC 290 (2.74 mg/100 mL), at 24 h by *L. acidophilus* 33200 (2.97 mg/100 mL), *L. acidophilus* 4461 (3.19 mg/100 mL) and *L. casei* 2607 (2.72 mg/100 mL) and at 36 h by *B. animalis* Bb12 (2.45 mg/100 mL) and *L. acidophilus* 4962 (2.94 mg/100 mL) from only 0.570 mg/100 mL at 0 hours of fermentation. Beyond 36 h of incubation, there was generally a reduced level of aglycone concentration, but significantly ($P < 0.05$) for incubation with *L. acidophilus* 33200 (Table 4.1) and *L. casei* 2607 (Table 4.3).

Genistein contributed the greatest concentration of bioactive isoflavone aglycones in each of the soymilk fermented by all the 6 microorganisms. This was evidently due to the higher

concentration of genistin in the original unfermented soymilk compared to the other β -glycosides isomers of daidzin and glycitin. It was also observed that daidzin occurred in much higher concentration compared to glycitin in the unfermented soymilk (that is, at 0 h). Consequently, the concentration of the aglycone forms (daidzein and glycitein) during fermentation of soymilk reflected proportionately the occurrence of the glycoside forms in the unfermented soymilk. In soymilk fermented by *L. acidophilus* 33200 (Table 4.1), there was a 7.0 fold increase in the concentration of genistein after 24 h of incubation with 90% of the original 2.199 mg per 100 mL genistin transformed into genistein. In comparison to the concentration of daidzein and glycitein, there was an increment of 3.1 and 3.7-fold, respectively after 24 h incubation. In this case, some 84% of the concentration of daidzein (0.848 mg per 100 mL at 0 h) and 28% of the concentration of glycitin (0.215 mg per 100 mL at 0 h) were transferred into their aglycone isomers. In overall, the total aglycone concentration increased 5.2 fold after 24 h incubation in fermented soymilk. This implies that some 84.5% of the total concentration of glycosides (3.262 mg per 100 mL at 0 h) was transformed into aglycone isomers. Tsangalis *et al.* (2002) had previously used ruptured cells of bifidobacteria and established the same trend of isoflavone transformation. Wang and Murphy (1996) in the manufacture of tempeh, used fungal β -glucosidase on cooked soybeans and noted a significant increase ($P < 0.05$) in the concentration of daidzein and genistein after 22 h of incubation at 37°C. Fermentation of cooked soybeans with *Rhizopus oligosporus* caused a 7.3 and 6.2 fold increase in the concentration of daidzein and genistein, respectively. The increase in aglycone concentrations was possibly due to the fungal enzymatic hydrolysis of isoflavone glycosides to form these aglycones and this is similar to the process occurring in soymilk fermented by probiotic *L. acidophilus*, *Bifidobacterium* and *L. casei* strains.

Comparatively, varying levels of isoflavone Glycoside hydrolysis (with increase in aglycone concentrations) occurred between soymilk fermented with *B. animalis* Bb12 (Table 4.2), *L. casei*

2607 (Table 4.3), *L. acidophilus* 4962 (Table 4.4), *L. acidophilus* 4461 (Table 4.5) and *L. casei* ASCC 290 (Table 4.6). As shown in Table 4.2, soymilk fermented with *B. animalis* Bb12 showed a significant increase ($P < 0.05$) in aglycone concentration from 0.570 mg per 100 mL (at 0 h) to a peak of 2.445 mg per 100 mL after 36 h of incubation. Some 81.6% of the isoflavone glycosides were hydrolysed into the aglycone form, a 4.3 fold increment. Tsangalis *et al.* (2002), using ruptured cells of *B. animalis* Bb12 in the fermentation of soymilk, found a 7.0 fold increase in the concentration of aglycones after 24 h of incubation.

The glycoside isoflavone components in soymilk fermented by *L. casei* 2607 reduced from 3.262 mg per 100 mL at 0 h to 0.593 mg per 100 mL after 24 h of incubation. In the same period, there was a significant increase in the concentration of isoflavone aglycones from 0.570 mg per 100 mL (at 0 h) to 2.718 mg per 100 mL (Table 4.3). The same trend of increment ($P < 0.05$) in aglycone concentrations during incubation and a concomitant reduction ($P < 0.05$) in β -glycoside concentration occurred for the other probiotics used as shown in Tables 4.4 to 4.6.

The greatest increase in the concentration of bioactive isoflavone aglycones occurred in soymilk fermented by *L. acidophilus* 4461 from 0.570 mg per 100 mL (at 0 h) to 3.174 per 100 mL after 24 h of incubation (Table 4.5). Of the isoflavone Glycosides, the concentration of β -glycosidic forms underwent the most significant transformation to aglycones especially in the presence of *L. acidophilus* 4461. Some 91% of 3.262 mg per 100 mL (at 0 h) were transformed into aglycone forms with the concentration of Glycosides reducing to 0.292 mg per 100 mL (at 24 h) incubation (Table 4.5). The maximum isoflavone aglycone concentration in soymilk at peak β -glucosidase activity with the 6 microorganisms ranged from 2.445 to 3.174 mg per 100 mL of soymilk (Tables 4.1 – 4.6).

4.3.4 Evaluation of the hydrolytic potential of probiotics in the isoflavone biotransformation process

As shown in Figures 4.2 to 4.7, there was a significant decrease ($P < 0.05$) in the concentration of isoflavone β -glycosides with a corresponding significant increase ($P < 0.05$) in the concentration of isoflavone aglycones during incubation with the micro-organisms. The breakdown of isoflavone glycosides into sugar moieties and bioactive isoflavone aglycones during incubation could be significant in improving the biological activity of soymilk. The lines representing an increase in isoflavone aglycones and a decrease in isoflavane β -glycosides crossed at a certain point during incubation. At this point, there was an equilibrium concentration of both isoflavone aglycones and isoflavone β -glycosides. For *L. acidophilus* and *L. casei*, this equilibrium cross-over point occurred before 12 h (Figs. 4.2, 4.4 to 4.7) while it occurred slightly after 24 h for *B. animalis* (Figure 4.3). The equilibrium point was important in determining the rate of isoflavone biotransformation and was an indicator of the β -glucosidase activity of the micro organisms during incubation. Consequently, the micro organisms that showed early equilibrium aglycone- β -glycosides cross-over point had a faster rate of glycoside hydrolysis and a higher β -glucosidase activity (units of enzyme) than those that showed a late cross-over point.

In order to evaluate the hydrolytic potential of each of the microorganisms in the biotransformation of isoflavones, it was important to observe the trend during incubation. As already discussed, the degree of biotransformation of isoflavone glycosides followed the trend of β -glucosidase activity (Table 3.2); hence it was reasonable to conclude that it was an enzymatic hydrolytic process. For each of the micro organism used during incubation in soymilk, the point at which an organism underwent exponential growth, peak β -glucosidase activity occurred. Concurrently, the same point of maximum enzyme activity also gave the maximum isoflavone aglycone concentration and minimum isoflavone β -glycoside concentration. In order to determine

the isoflavone hydrolytic potential, it was important to calculate the isoflavone β -glycoside reduction quotient and the isoflavone aglycone increment quotient at the point of peak β -glucosidase activity during incubation. Using the two ratios, a new isoflavone hydrolytic index (proposed to be known as Otieno - Shah index), was calculated. The values of Otieno – Shah (O–S) index reflected the isoflavone hydrolytic potential of the micro-organisms for the biotransformation of isoflavone β -glycosides. As shown in Table 4.7, the O-S index can be used to determine the hydrolytic potential of each microorganism for the biotransformation of isoflavones and could be used to analyse the hydrolytic potential of a micro-organism. The higher the hydrolytic index (O–S index), the better was the hydrolytic potential of that microorganism. Although no research has been carried out in evaluating the isoflavone hydrolytic potential of a wider range of probiotic microorganisms for the biotransformation of isoflavone glycosides, it is reasonable to assume that an O-S index of 1.0 or more would be acceptable. An O-S index of 1.0 equates to 35-45% decrease in the total isoflavone β -glycoside concentration and a concurrent increase of at least 40-42% in total aglycone concentration at peak β -glucosidase activity. *L. acidophilus* 4461 had the highest hydrolytic potential with an O-S index of 2.01 at 24 h of incubation at 37°C while *L. casei* 2607 had the least O-S index of 1.15 at 24 h of incubation at 37°C.

4.4 CONCLUSIONS

All the 6 microorganisms produced varying levels of β -glucosidase activity for enzyme-induced biotransformation of the isoflavone β -glycosides depending on the stage of incubation. The enzyme activity and the hydrolytic ability were unique for each strain. There was a progressive decrease in the concentration of β -glycosides as well as a gradual increase in the isoflavone aglycone concentrations. The level of aglycones increased from 8% in the non-fermented soymilk

to a peak concentration value of 60.3 to 76.9%, while the concentration of isoflavone β -glycosides decreased from 83% in the non-fermented soymilk to 8.33 to 14.5% at peak β -glucosidase activity. The equilibrium point between isoflavone aglycone and isoflavone β -glycoside concentration during incubation was indicative of the hydrolytic potential of β -glucosidase from each microorganism. *L. acidophilus* 4461 had the greatest hydrolytic potential of isoflavone glycosides in soymilk. *L. acidophilus* strains had a higher hydrolytic potential than *B. animalis* followed by *L. casei* strains. In addition to being probiotic and influencing the intestinal health, these bacteria could be used to alter the biological activity of soymilk by transforming the predominant concentration of isoflavone glycosides to bioactive aglycones.

Table 4.1 Concentration of isoflavone isomers (mg/ 100 mL) in soymilk fermented by *L. acidophilus* 33200 for 12, 24, 36 and 48 h of incubation at 37°C

Isoflavone isomer	Incubation Time										
	0 h		12 h		24 h		36 h		48 h		P- Value
Glycitin	0.215 ^a ± 0.045		0.209 ^a ± 0.046		0.154 ^a ± 0.041		0.259 ^a ± 0.071		0.168 ^a ± 0.034		
Daidzin	0.848 ^a ± 0.192		0.194 ^b ± 0.011		0.136 ^b ± 0.055		0.162 ^b ± 0.042		0.138 ^b ± 0.020		0.0003*
Genistin	2.199 ^a ± 0.006		0.416 ^b ± 0.056		0.215 ^b ± 0.026		0.291 ^b ± 0.034		0.287 ^b ± 0.084		1.04E-08*
β-Glycosides ¹	3.262 ^a ± 0.153		0.818 ^b ± 0.099		0.505 ^b ± 0.117		0.712 ^b ± 0.016		0.593 ^b ± 0.131		2.83E-07*
Glycitein	0.047 ^b ± 0.001		0.212 ^a ± 0.014		0.175 ^a ± 0.014		0.180 ^a ± 0.005		0.172 ^a ± 0.015		0.0001*
Daidzein	0.224 ^b ± 0.079		0.780 ^a ± 0.047		0.703 ^a ± 0.029		0.688 ^a ± 0.017		0.588 ^a ± 0.077		0.0006*
Genistein	0.299 ^b ± 0.095		1.536 ^{ac} ± 0.055		2.093 ^{ac} ± 0.237		1.751 ^{ac} ± 0.063		0.872 ^{bc} ± 0.199		0.0002*
Aglycones ¹	0.570 ^b ± 0.175		2.528 ^a ± 0.069		2.971 ^a ± 0.215		2.619 ^a ± 0.043		1.632 ^{ac} ± 0.216		3.05E-05*
Malonylglycitin	0.055 ^b ± 0.000		0.053 ^a ± 0.034		0.066 ^a ± 0.005		0.106 ^a ± 0.026		0.200 ^a ± 0.065		0.0965
Malonydaidzin	0.018 ^a ± 0.002		0.082 ^b ± 0.018		0.050 ^b ± 0.008		0.107 ^{bc} ± 0.017		0.215 ^{ac} ± 0.039		0.0019*
Malonylgenistin	0.039 ^a ± 0.004		0.091 ^a ± 0.039		0.132 ^a ± 0.057		0.084 ^a ± 0.020		0.152 ^a ± 0.037		0.4082
Malonylglycosides ¹	0.111 ^a ± 0.006		0.226 ^{bc} ± 0.054		0.248 ^{bc} ± 0.049		0.297 ^{bc} ± 0.006		0.568 ^{ac} ± 0.135		0.0220*
Acetylglycitin	0.057 ^a ± 0.015		0.273 ^b ± 0.018		0.173 ^b ± 0.052		0.157 ^b ± 0.050		0.457 ^{ab} ± 0.077		0.0051*
Acetydaidzin	0.092 ^a ± 0.010		0.135 ^a ± 0.019		0.106 ^a ± 0.044		0.138 ^a ± 0.040		0.324 ^a ± 0.060		0.0205*
Acetylgenistin	0.047 ^a ± 0.034		0.161 ^b ± 0.019		0.138 ^b ± 0.044		0.218 ^b ± 0.034		0.566 ^a ± 0.111		0.0020*
Acetylglycosides ¹	0.196 ^b ± 0.028		0.568 ^{bc} ± 0.014		0.417 ^{bc} ± 0.113		0.513 ^{bc} ± 0.057		1.347 ^a ± 0.214		0.0009*
Equol	ND		ND		ND		ND		ND		N/A
Total Isoflavones ²	4.140 ^a ± 0.001		4.140 ^a ± 3.44E-08		1.0000						

Mean ± standard error, two trials in triplicates (n = 6). Means in the same row with different superscripts are 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

¹Mean total of 3 respective isomers

²Mean total of malonyl-, acetyl-, β-glycoside, aglycone, and equol isomers

One-way ANOVA of means in the same row

*Significant difference (P < 0.05) – is the concentration of the isoflavone isomer during the 48 h fermentation period

Table 4.2 Concentration of isoflavone isomers (mg/ 100 mL) in soymilk fermented by *B. animalis* Bb12 for 12, 24, 36 and 48 h of incubation at 37°C

Isoflavone isomer	Incubation Time										P- Value
	0 h		12 h		24 h		36 h		48 h		
Glycitin	0.215 ^a ± 0.045		0.520 ^a ± 0.298		0.670 ^a ± 0.455		0.162 ^a ± 0.017		0.145 ^a ± 0.007		0.5362
Daidzin	0.848 ^a ± 0.192		0.422 ^a ± 0.197		0.804 ^a ± 0.334		0.155 ^a ± 0.025		0.125 ^a ± 0.008		0.0833
Genistin	2.199 ^a ± 0.006		1.877 ^a ± 0.085		0.099 ^b ± 0.024		0.283 ^b ± 0.050		0.187 ^b ± 0.066		2.1E-09*
β-Glycosides ¹	3.262 ^a ± 0.153		2.819 ^a ± 0.198		1.573 ^b ± 0.169		0.600 ^{bc} ± 0.043		0.458 ^{bc} ± 0.056		5.36E-07*
Glycitein	0.047 ^b ± 0.001		0.062 ^{bc} ± 0.009		0.120 ^{ac} ± 0.033		0.210 ^a ± 0.012		0.189 ^a ± 0.013		0.0005*
Daidzein	0.224 ^b ± 0.079		0.190 ^b ± 0.021		0.444 ^{bc} ± 0.041		0.797 ^a ± 0.046		0.752 ^a ± 0.051		1.2E-05*
Genistein	0.299 ^b ± 0.095		0.294 ^b ± 0.059		0.839 ^{ac} ± 0.051		1.438 ^a ± 0.105		1.460 ^a ± 0.162		3.46E-05*
Aglycones ¹	0.570 ^b ± 0.175		0.546 ^b ± 0.071		1.403 ^{bc} ± 0.115		2.445 ^a ± 0.162		2.401 ^a ± 0.224		1.85E-05*
Malonylglycitin	0.055 ^b ± 0.000		0.053 ^b ± 0.020		0.182 ^{ac} ± 0.011		0.148 ^{bc} ± 0.024		0.111 ^{bc} ± 0.017		0.0027*
Malonydaidzin	0.018 ^a ± 0.002		0.139 ^a ± 0.072		0.103 ^a ± 0.010		0.124 ^a ± 0.019		0.093 ^a ± 0.007		0.3337
Malonylgenistin	0.039 ^a ± 0.004		0.111 ^a ± 0.057		0.219 ^a ± 0.044		0.082 ^a ± 0.006		0.099 ^a ± 0.017		0.0632
Malonylglycosides ¹	0.111 ^a ± 0.006		0.303 ^a ± 0.136		0.504 ^a ± 0.040		0.353 ^a ± 0.038		0.303 ^a ± 0.028		0.0663
Acetylglycitin	0.057 ^a ± 0.015		0.205 ^a ± 0.058		0.283 ^a ± 0.103		0.225 ^a ± 0.041		0.236 ^a ± 0.109		0.5069
Acetydaidzin	0.092 ^a ± 0.010		0.181 ^a ± 0.051		0.137 ^a ± 0.025		0.184 ^a ± 0.023		0.228 ^a ± 0.085		0.5066
Acetylgenistin	0.047 ^a ± 0.034		0.086 ^a ± 0.017		0.240 ^a ± 0.051		0.333 ^a ± 0.111		0.515 ^a ± 0.152		0.0444*
Acetylglycosides ¹	0.196 ^a ± 0.028		0.472 ^a ± 0.093		0.660 ^a ± 0.178		0.742 ^a ± 0.092		0.978 ^a ± 0.282		0.1183
Equol	ND		ND		ND		ND		ND		N/A
Total Isoflavones ²	4.140 ^a ± 0.001		4.140 ^a ± 3.44E-08		1.0000						

Mean ± standard error, two trials in triplicates (n = 6). Means in the same row with different superscripts are 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

¹Mean total of 3 respective isomers

²Mean total of malonyl-, acetyl-, β-glycoside, aglycone, and equol isomers

One-way ANOVA of means in the same row

*Significant difference (P < 0.05) – is the concentration of the isoflavone isomer during the 48 h fermentation period

Table 4.3 Concentration of isoflavone isomers (mg/ 100 mL) in soymilk fermented by *L. casei* 2607 for 12, 24, 36 and 48 h of incubation at 37°C

Isoflavone isomers	Incubation Time										
	0 h		12 h		24 h		36 h		48 h		P- Value
Glycitin	0.215 ^a ±	0.045	0.378 ^a ±	0.094	0.125 ^a ±	0.004	0.147 ^a ±	0.026	0.344 ^a ±	0.068	
Daidzin	0.848 ^a ±	0.192	0.256 ^b ±	0.103	0.187 ^b ±	0.086	0.161 ^b ±	0.014	0.364 ^{ab} ±	0.096	0.0090*
Genistin	2.199 ^a ±	0.006	0.569 ^b ±	0.294	0.281 ^b ±	0.042	0.186 ^b ±	0.070	0.197 ^b ±	0.042	6.4E-05*
β-Glycosides ¹	3.262 ^a ±	0.153	1.202 ^b ±	0.471	0.593 ^b ±	0.093	0.494 ^b ±	0.107	0.905 ^b ±	0.165	0.0005*
Glycitein	0.047 ^b ±	0.001	0.178 ^b ±	0.047	0.205 ^b ±	0.001	0.222 ^b ±	0.020	0.262 ^{ab} ±	0.054	0.0367*
Daidzein	0.224 ^b ±	0.079	0.475 ^{ab} ±	0.090	0.797 ^a ±	0.020	0.854 ^a ±	0.074	0.543 ^{ab} ±	0.079	0.0017*
Genistein	0.299 ^b ±	0.095	1.328 ^b ±	0.399	1.716 ^{ab} ±	0.056	1.525 ^b ±	0.122	0.789 ^b ±	0.234	0.0183*
Aglycones ¹	0.570 ^b ±	0.175	1.981 ^b ±	0.503	2.718 ^{ab} ±	0.046	2.600 ^{ab} ±	0.055	1.594 ^b ±	0.316	0.0065
Malonylglycitin	0.055 ^b ±	0.000	0.057 ^b ±	0.037	0.117 ^{bc} ±	0.011	0.185 ^{bc} ±	0.027	0.240 ^{ac} ±	0.017	0.0018*
Malonydaidzin	0.018 ^b ±	0.002	0.125 ^{bc} ±	0.057	0.085 ^{bc} ±	0.009	0.163 ^{bc} ±	0.024	0.211 ^{ac} ±	0.015	0.0222*
Malonylgenistin	0.039 ^a ±	0.004	0.119 ^a ±	0.039	0.137 ^a ±	0.034	0.144 ^a ±	0.010	0.167 ^a ±	0.012	0.0927
Malonylglycosides ¹	0.111 ^b ±	0.006	0.301 ^{ab} ±	0.101	0.339 ^{ab} ±	0.043	0.493 ^a ±	0.058	0.619 ^a ±	0.044	0.0046*
Acetylglycitin	0.057 ^b ±	0.015	0.200 ^{bc} ±	0.021	0.163 ^{bc} ±	0.013	0.159 ^{bc} ±	0.041	0.325 ^{ac} ±	0.051	0.0068
Acetydaidzin	0.092 ^a ±	0.010	0.283 ^a ±	0.119	0.113 ^a ±	0.014	0.164 ^a ±	0.012	0.245 ^a ±	0.055	0.2629
Acetylgenistin	0.047 ^b ±	0.034	0.173 ^{bc} ±	0.065	0.213 ^{bc} ±	0.076	0.231 ^{bc} ±	0.050	0.453 ^{ac} ±	0.071	0.0249*
Acetylglycosides ¹	0.196 ^b ±	0.028	0.656 ^{bc} ±	0.160	0.490 ^{bc} ±	0.093	0.554 ^{bc} ±	0.065	1.022 ^{ac} ±	0.164	0.0188*
Equol	ND		ND		ND		ND		ND		N/A
Total Isoflavones ²	4.140 ^a ±	0	4.140 ^a ±	3.441E-08	1.0000						

Mean ± standard error, two trials in triplicates (n = 6). Means in the same row with different superscripts are 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

¹Mean total of 3 respective isomers

²Mean total of malonyl-, acetyl-, β-Glycoside, aglycone, and equol isomers

One-way ANOVA of means in the same row

*Significant difference (P < 0.05) – is the concentration of the isoflavone isomer during the 48 h fermentation period

Table 4.4 Concentration of isoflavone isomers (mg/ 100 mL) in soymilk fermented by *L. acidophilus* 4962 for 12, 24, 36 and 48 h of incubation at 37°C

Isoflavone isomer	Incubation Time										
	0 h		12 h		24 h		36 h		48 h		P- Value
Glycitin	0.215 ^a ± 0.045		0.172 ^a ± 0.064		0.112 ^a ± 0.015		0.072 ^a ± 0.005		0.097 ^a ± 0.012		
Daidzin	0.848 ^a ± 0.192		0.185 ^b ± 0.043		0.077 ^b ± 0.009		0.117 ^b ± 0.042		0.152 ^b ± 0.033		0.0002*
Genistin	2.199 ^a ± 0.006		0.22 ^{3b} ± 0.029		0.171 ^b ± 0.066		0.156 ^b ± 0.054		0.152 ^b ± 0.069		8.14E-09*
β-Glycosides ¹	3.262 ^a ± 0.153		0.581 ^b ± 0.096		0.360 ^b ± 0.081		0.345 ^b ± 0.093		0.401 ^b ± 0.100		7.29E-08*
Glycitein	0.047 ^b ± 0.001		0.213 ^a ± 0.007		0.190 ^a ± 0.007		0.222 ^a ± 0.012		0.212 ^a ± 0.008		2.76E-06*
Daidzein	0.224 ^b ± 0.079		0.811 ^a ± 0.022		0.774 ^a ± 0.045		0.871 ^a ± 0.042		0.785 ^a ± 0.030		2.67E-05*
Genistein	0.299 ^b ± 0.095		1.683 ^a ± 0.052		1.902 ^a ± 0.177		1.851 ^a ± 0.271		1.579 ^a ± 0.200		0.0027*
Aglycones ¹	0.570 ^b ± 0.175		2.707 ^a ± 0.037		2.866 ^a ± 0.208		2.944 ^a ± 0.324		2.577 ^a ± 0.234		0.0005*
Malonylglycitin	0.055 ^a ± 0.000		0.090 ^a ± 0.042		0.110 ^a ± 0.012		0.096 ^a ± 0.016		0.118 ^a ± 0.009		0.4843
Malonydaidzin	0.018 ^a ± 0.002		0.110 ^a ± 0.036		0.120 ^a ± 0.049		0.113 ^a ± 0.041		0.126 ^a ± 0.061		0.5920
Malonylgenistin	0.039 ^a ± 0.004		0.077 ^a ± 0.019		0.156 ^a ± 0.024		0.086 ^a ± 0.028		0.101 ^a ± 0.022		0.0635
Malonylglycosides ¹	0.111 ^a ± 0.006		0.277 ^a ± 0.094		0.387 ^a ± 0.064		0.295 ^a ± 0.084		0.345 ^a ± 0.091		0.3246
Acetylglycitin	0.057 ^a ± 0.015		0.224 ^a ± 0.016		0.208 ^a ± 0.043		0.254 ^a ± 0.113		0.248 ^a ± 0.125		0.6108
Acetydaidzin	0.092 ^a ± 0.010		0.130 ^a ± 0.019		0.129 ^a ± 0.021		0.110 ^a ± 0.028		0.140 ^a ± 0.016		0.6222
Acetylgenistin	0.047 ^a ± 0.034		0.221 ^a ± 0.085		0.191 ^a ± 0.069		0.193 ^a ± 0.059		0.429 ^a ± 0.090		0.0769
Acetylglycosides ¹	0.196 ^b ± 0.028		0.576 ^{abc} ± 0.067		0.527 ^{abc} ± 0.075		0.557 ^{abc} ± 0.164		0.817 ^{ac} ± 0.045		0.0295*
Equol	ND		ND		ND		ND		ND		N/A
Total Isoflavones ²	4.140 ^a ± 0		4.140 ^a ± 3.44E-08		1.0000						

Mean ± standard error, two trials in triplicates (n = 6). Means in the same row with different superscripts are 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

¹Mean total of 3 respective isomers

²Mean total of malonyl-, acetyl-, β-Glycoside, aglycone, and equol isomers

One-way ANOVA of means in the same row

*Significant difference (P < 0.05) – is the concentration of the isoflavone isomer during the 48 h fermentation period

Table 4.5 Concentration of isoflavone isomers (mg/ 100 mL) in soymilk fermented by *L. acidophilus* 4461 for 12, 24, 36 and 48 h of incubation at 37°C

Isoflavone isomers	Incubation Time										P- Value
	0 h		12 h		24 h		36 h		48 h		
Glycitin	0.215 ^a ±	0.045	0.128 ^a ±	0.022	0.091 ^a ±	0.022	0.097 ^a ±	0.021	0.114 ^a ±	0.011	0.0456*
Daidzin	0.848 ^a ±	0.192	0.123 ^b ±	0.028	0.074 ^b ±	0.010	0.073 ^b ±	0.011	0.071 ^b ±	0.002	4.16E-05*
Genistin	2.199 ^a ±	0.006	0.127 ^b ±	0.016	0.127 ^b ±	0.007	0.106 ^b ±	0.025	0.098 ^b ±	0.009	7.07E-14*
β-Glycosides ¹	3.262 ^a ±	0.153	0.377 ^b ±	0.048	0.292 ^b ±	0.033	0.277 ^b ±	0.036	0.283 ^b ±	0.003	2.32E-10*
Glycitein	0.047 ^b ±	0.001	0.215 ^a ±	0.015	0.214 ^a ±	0.021	0.202 ^a ±	0.031	0.216 ^a ±	0.027	0.0065
Daidzein	0.224 ^b ±	0.079	0.869 ^a ±	0.044	0.830 ^a ±	0.054	0.849 ^a ±	0.101	0.914 ^a ±	0.015	0.0005*
Genistein	0.299 ^b ±	0.095	2.052 ^a ±	0.129	2.129 ^a ±	0.109	2.134 ^a ±	0.109	1.797 ^a ±	0.006	5.31E-06*
Aglycones	0.570 ^b ±	0.175	3.136 ^a ±	0.117	3.174 ^a ±	0.110	3.185 ^a ±	0.127	2.927 ^a ±	0.042	5.56E-07*
Malonylglycitin	0.055 ^a ±	0.000	0.050 ^a ±	0.022	0.087 ^a ±	0.003	0.111 ^a ±	0.020	0.123 ^a ±	0.016	0.0438*
Malonydaidzin	0.018 ^b ±	0.002	0.090 ^a ±	0.012	0.082 ^a ±	0.015	0.082 ^a ±	0.004	0.118 ^a ±	0.009	0.0024*
Malonylgenistin	0.039 ^{bc} ±	0.004	0.066 ^{bc} ±	0.002	0.107 ^{ac} ±	0.020	0.055 ^{bc} ±	0.007	0.073 ^{bc} ±	0.010	0.0266*
Malonylglycosides	0.111 ^b ±	0.006	0.205 ^a ±	0.016	0.275 ^a ±	0.033	0.248 ^a ±	0.027	0.314 ^a ±	0.014	0.0028*
Acetylglycitin	0.057 ^b ±	0.015	0.138 ^{bc} ±	0.023	0.161 ^{bc} ±	0.040	0.113 ^{bc} ±	0.025	0.246 ^{ac} ±	0.039	0.0322*
Acetyldaizin	0.092 ^a ±	0.010	0.122 ^a ±	0.020	0.128 ^a ±	0.026	0.091 ^a ±	0.014	0.153 ^a ±	0.032	0.3707
Acetylgenistin	0.047 ^a ±	0.034	0.162 ^a ±	0.074	0.110 ^a ±	0.021	0.226 ^a ±	0.096	0.217 ^a ±	0.014	0.3306
Acetylglycosides	0.196 ^a ±	0.028	0.422 ^a ±	0.107	0.399 ^a ±	0.078	0.430 ^a ±	0.126	0.616 ^a ±	0.048	0.1428
Equol	ND		ND		ND		ND		ND		N/A
Total Isoflavones	4.140 ^a ±	0	4.140 ^a ±	3.44E-08	1.0000						

Mean ± standard error, two trials in triplicates (n = 6). Means in the same row with different superscripts are 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

¹Mean total of 3 respective isomers

²Mean total of malonyl-, acetyl-, β-Glycoside, aglycone, and equol isomers

One-way ANOVA of means in the same row

*Significant difference (P < 0.05) – is the concentration of the isoflavone isomer during the 48 h fermentation period

Table 4.6 Concentration of isoflavone isomers (mg/ 100 mL) in soymilk fermented by *L. casei* ASCC 290 for 12, 24, 36 and 48 h of incubation at 37°

Isoflavone isomers	Incubation Time										
	0 h		12 h		24 h		36 h		48 h		P- Value
Glycitin	0.215 ^a ± 0.045		0.167 ^a ± 0.064		0.128 ^a ± 0.019		0.138 ^a ± 0.030		0.101 ^a ± 0.021		
Daidzin	0.848 ^a ± 0.192		0.113 ^b ± 0.039		0.193 ^b ± 0.085		0.133 ^b ± 0.014		0.126 ^b ± 0.036		0.0006*
Genistin	2.199 ^a ± 0.006		0.284 ^b ± 0.068		0.318 ^b ± 0.084		0.279 ^b ± 0.046		0.266 ^b ± 0.061		0.0000*
β-Glycosides ¹	3.262 ^a ± 0.153		0.564 ^b ± 0.157		0.639 ^b ± 0.165		0.550 ^b ± 0.041		0.493 ^b ± 0.077		0.0000*
Glycitein	0.047 ^b ± 0.001		0.202 ^{ab} ± 0.003		0.197 ^b ± 0.009		0.224 ^a ± 0.003		0.199 ^a ± 0.002		0.0000*
Daidzein	0.224 ^b ± 0.079		0.780 ^a ± 0.022		0.704 ^a ± 0.021		0.853 ^a ± 0.025		0.753 ^a ± 0.012		0.0000*
Genistein	0.299 ^b ± 0.095		1.760 ^a ± 0.128		1.647 ^a ± 0.192		1.622 ^a ± 0.177		1.744 ^a ± 0.127		0.0011*
Aglycones ¹	0.570 ^b ± 0.175		2.742 ^a ± 0.147		2.549 ^a ± 0.200		2.699 ^a ± 0.191		2.696 ^a ± 0.137		0.0001*
Malonylglycitin	0.055 ^a ± 0.000		0.080 ^a ± 0.035		0.096 ^a ± 0.008		0.125 ^a ± 0.027		0.134 ^a ± 0.025		0.2798
Malonydaidzin	0.018 ^b ± 0.002		0.110 ^a ± 0.023		0.133 ^a ± 0.056		0.110 ^a ± 0.015		0.110 ^a ± 0.019		0.2621
Malonylgenistin	0.039 ^{bc} ± 0.004		0.075 ^a ± 0.014		0.117 ^a ± 0.010		0.116 ^a ± 0.032		0.116 ^a ± 0.007		0.0710
Malonylglycosides ¹	0.111 ^b ± 0.006		0.265 ^a ± 0.061		0.346 ^a ± 0.058		0.350 ^a ± 0.033		0.360 ^a ± 0.037		0.0419*
Acetylglycitin	0.057 ^b ± 0.015		0.198 ^a ± 0.024		0.235 ^a ± 0.018		0.187 ^{ab} ± 0.035		0.153 ^{ab} ± 0.013		0.0092*
Acetyldaizn	0.092 ^a ± 0.010		0.172 ^a ± 0.008		0.193 ^a ± 0.024		0.143 ^a ± 0.016		0.169 ^a ± 0.079		0.5949
Acetylgenistin	0.047 ^a ± 0.034		0.199 ^a ± 0.066		0.179 ^a ± 0.051		0.211 ^a ± 0.098		0.270 ^a ± 0.057		0.3943
Acetylglycosides ¹	0.196 ^a ± 0.028		0.569 ^a ± 0.061		0.607 ^a ± 0.073		0.541 ^a ± 0.148		0.591 ^a ± 0.059		0.0947
Equol	ND		ND		ND		ND		ND		N/A
Total Isoflavones ²	4.140 ^a ± 0		4.140 ^a ± 3.44E-08		1.0000						

Mean ± standard error, two trials in triplicates (n = 6). Means in the same row with different superscripts are 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

¹Mean total of 3 respective isomers

²Mean total of malonyl-, acetyl-, β-Glycoside, aglycone, and equol isomers

One-way ANOVA of means in the same row

*Significant difference (P < 0.05) – is the concentration of the isoflavone isomer during the 48 h fermentation period

Table 4.7 Otieno – Shah (O –S) index (Isoflavone hydrolytic index) of 6 micro organisms in soymilk at peak β -glucosidase activity during incubation at 37°C

Micro-organisms	¹ β -Glycoside Reduction quotient	² Aglycone Increment quotient	³ Otieno-Shah Index	Ref. Table
<i>L. acidophilus</i> 33200	6.46	5.21	1.24 _{24h}	Table 4.1
<i>B. animalis</i> BB12	5.44	4.29	1.27 _{36h}	Table 4.2
<i>L. casei</i> 2607	5.50	4.77	1.15 _{24h}	Table 4.3
<i>L. acidophilus</i> 4962	9.06	5.02	1.81 _{24h}	Table 4.4
<i>L. acidophilus</i> 4461	11.17	5.57	2.01 _{24h}	Table 4.5
<i>L. casei</i> ASCC 290	5.78	4.81	1.20 _{12h}	Table 4.6

¹ β -Glycoside reduction quotient- refers to the ratio of initial isoflavone β -Glycoside concentration (at 0 h) in mg per 100mL of soymilk to the concentration of β -Glycosides during peak β -glucosidase activity of the micro organism during incubation at 37°C.

²Aglycone increment quotient- refers to the ratio of the concentration of bioactive isoflavone aglycones in mg per 100mL of soymilk during peak β -glucosidase activity to the initial concentration of aglycones (at 0 h) of the micro organism during incubation at 37°C.

³Otieno-Shah (O-S) index (isoflavone hydrolytic index)- refers to the ratio of β -Glycoside reduction quotient to the aglycone increment quotient

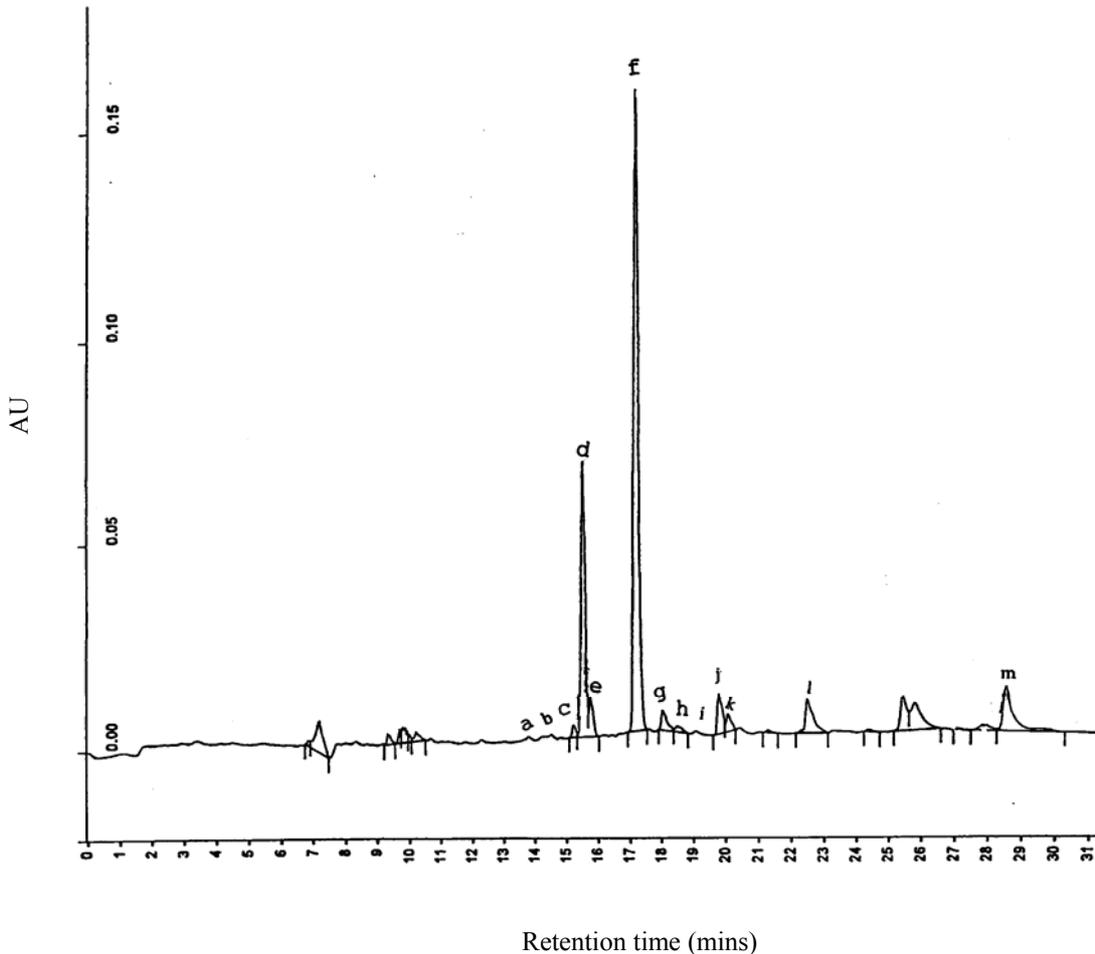


Figure 4.1. Reversed-phase HPLC chromatogram showing approximate retention times of isoflavone isomer metabolites (at wavelength of 260 nm) found in unfermented soymilk; a: malonyldaidzin (14.01 min), b: malonyglycitin (14.56 min), c: malonylgenistin (15.23 min), d: daidzin (15.52 min), e: glycitin (15.78 min), f: genistin (17.176 min), g: acetyldaidzin (18.06 min), h: acetyldaidzin (18.52 min), i: acetylgenistin (18.92 min), j: daidzein (19.82 min), k: glycitein (20.11 min), l: genistein (22.53 min), m: internal standard (ISTD) flavone (28.63 min).

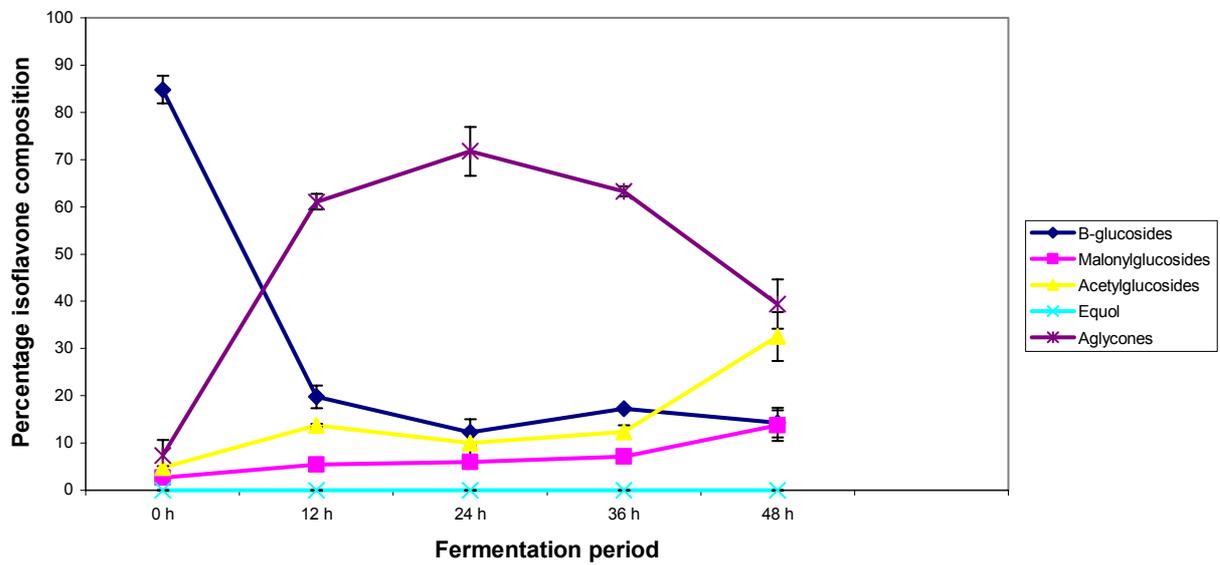


Figure 4.2 Percentage change in isoflavone composition during fermentation using *L. acidophilus* 33200 at 37°C.

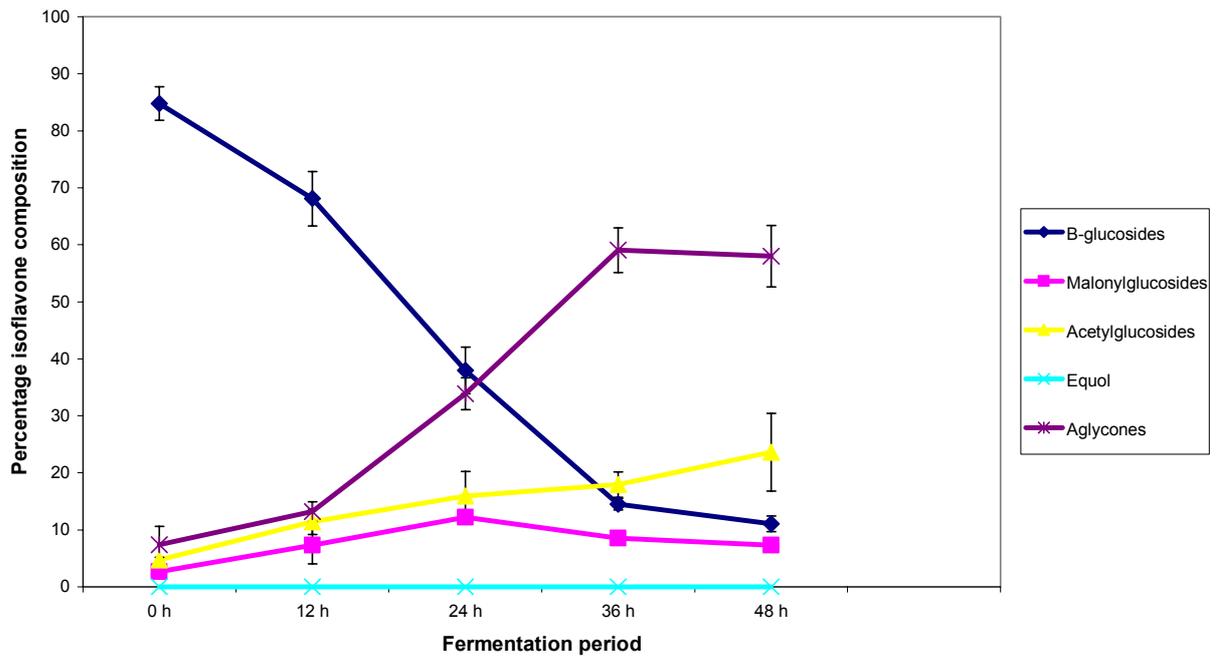


Figure 4.3 Percentage change in isoflavone composition during fermentation with *B. animalis* sp. *lactis* Bb12 at 37°C.

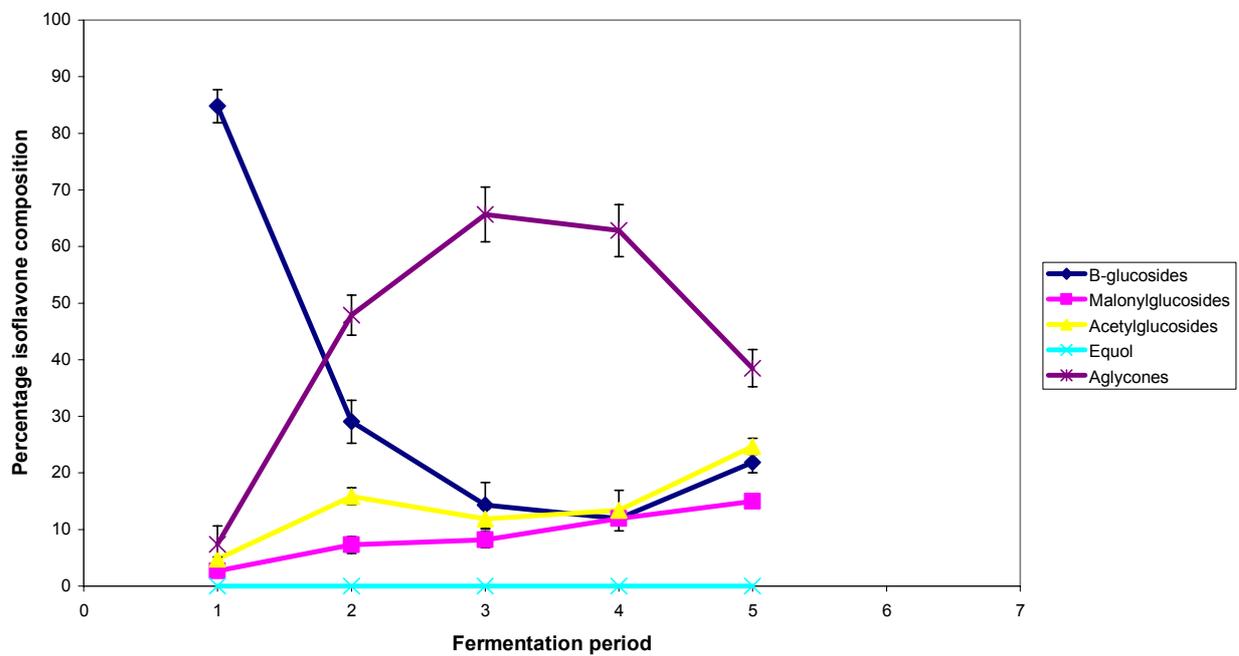


Figure 4.4 Percentage change in isoflavone composition during fermentation using *L. casei* 2607 at 37°C.

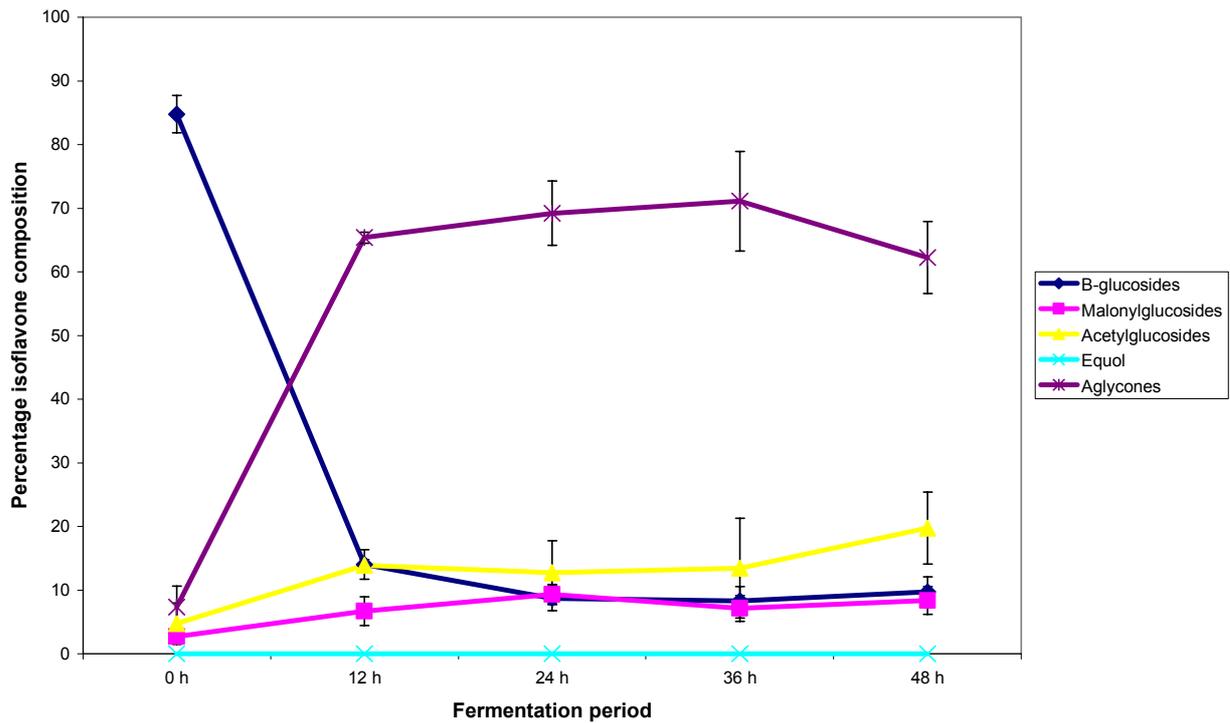


Figure 4.5 Percentage change in isoflavone composition during fermentation using *L. acidophilus* 4962 at 37°C.

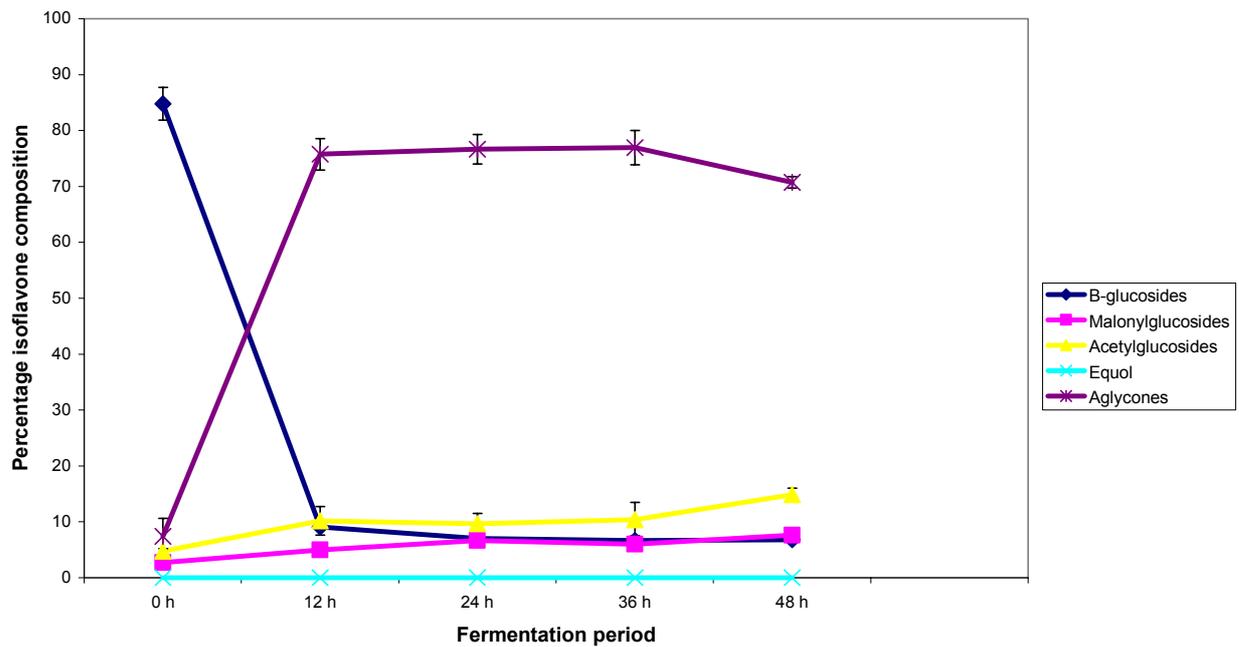


Figure 4.6 Percentage change in isoflavone composition during fermentation using *L. acidophilus* 4461 at 37°C.

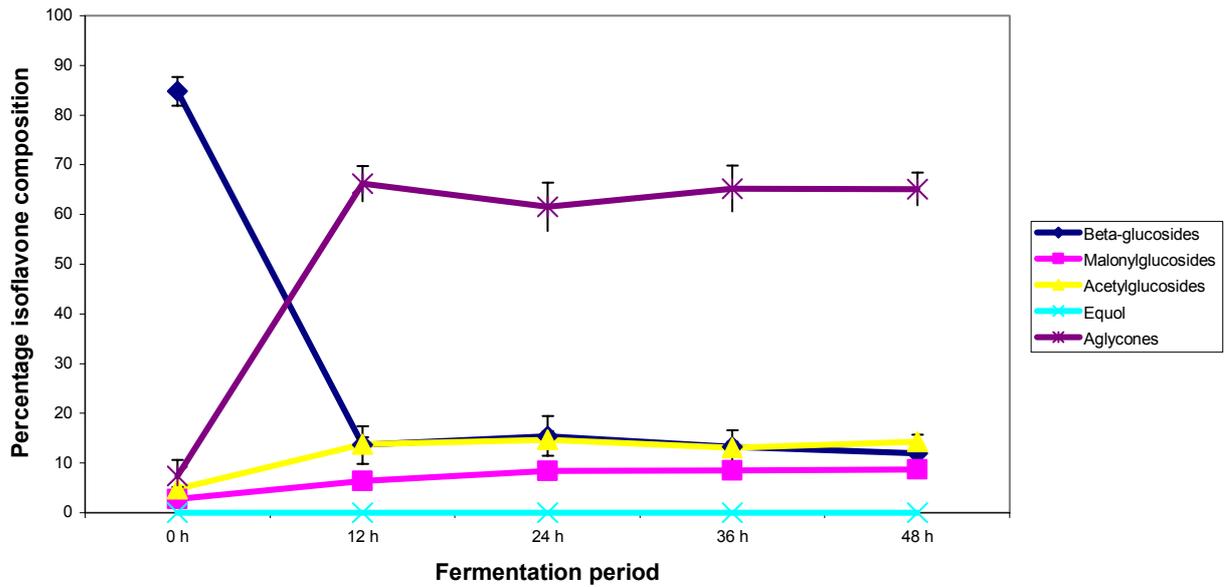


Figure 4.7 Percentage change in isoflavone composition during fermentation with *L. casei* ASCC 290 at 37°C.

5.0 Stability of isoflavone phytoestrogens in fermented soymilk with *Bifidobacterium animalis* ssp. *lactis* Bb12 during storage at different temperatures

5.1 INTRODUCTION

Isoflavones are intrinsic plant compounds having a 1, 2 – diarylpropane structure. Structurally, they are well defined and to date, over 230 members of the class have been identified (Cassidy *et al.* 2000). They are an isomeric class of flavonoids found abundantly in soybeans. The parent isoflavones are the aglycone structures of daidzein, genistein and glycitein, which are conjugated to form malonyl-, acetyl- and β -Glycoside configurations. The aglycones are structurally similar to the mammalian oestrogen oestradiol-17 β and therefore mimic the function of estradiol in the human body (Setchell & Cassidy, 1999). Due to the structural similarity, they share several features in common with estradiol, including a pair of hydroxyl groups separated by a similar distance (Miksicek, 1995) and the presence of a phenolic ring which is a prerequisite for binding into the oestrogen receptor (Metzger *et al.* 1995). However, small differences in structure of the individual phytoestrogens can dramatically alter estrogenicity. The isoflavones, daidzein and genistein for example, share identical structures except for an additional group on the A ring of genistein but genistein may have up to five- or six-fold greater oestrogenic activity in some assay systems (Setchell & Cassidy, 1999).

Many animal and human clinical studies, but mostly epidemiological studies (usually comparing the occurrence of a certain disease in Asian countries with Western countries), suggest the existence of preventative effects of soy consumption for several hormone-dependent diseases (Setchell, 2000). Some of the health benefits are prevention of breast-, prostate- and colon cancers, cardiovascular disease, bone health problems, and postmenopausal symptoms.

Isoflavones are not generally destroyed by heat but are rather subject to intra-conversion between different forms (Setchell *et al.* 1998).

However, these studies have only been limited to total isoflavone losses during each of the processes. The stability of the individual bioactive isoflavone aglycone components has not been studied during storage at lower temperatures. In our previous study (Chapter 3.0), we reported that microbial β -glucosidases were responsible for an increase in isoflavone aglycone components in fermented soymilk. It is important that once the biological activity of a soy-based product has been improved, the stability of bioactive components be maintained during the storage period in order to confer the potential health benefits to the consumers. To date, very little work has been conducted in the assessment of changes in isoflavone profile during storage and little data are available on the kinetics of isoflavone degradation. Such data are crucial for the assessment of the health potential of soy foods. Hence, the objectives of this study were to examine the stability of the bioactive isoflavone aglycones in fermented soymilk made from soy protein isolate (SPI) during storage at various temperatures (-80°C, 4°C, 25°C and 37°C) and to establish the degradation constants for each isoflavone isomer during storage at these storage temperatures.

5.2 MATERIALS AND METHODS

5.2.1 Bacteria

Pure culture of *B.animalis* ssp. *lactis* Bb12 was obtained from Chr Hansen Pty. Ltd. (Bayswater, Vic., Australia) and the purity of the culture was checked and stored as described in section 3.2.1.

5.2.2 Bacterial growth media

Rehydrated de Mann Rogosa Sharpe (MRS) broth (pH adjusted to 6.7 using 5 M sodium hydroxide) (de Mann *et al.* 1960) was prepared as described in section 4.2.2.

5.2.3 Soymilk manufacture

Soy protein isolate (SPI; SUPRO 590), supplied by The Solae Co. (Chatswood, NSW, Australia), was reconstituted, autoclaved and pH adjusted as described in section 3.2.2.

5.2.4 Fermentation of soymilk with *B. animalis ssp. lactis* Bb12

The micro-organism was activated in MRS broth as outlined in section 3.2.3. In order to conduct storage studies, 4 bottles each containing 400 mL of sterile soymilk was inoculated (in duplicate) with the active culture (5% v/v), and incubated at 37°C for 24 h to achieve peak β -glucosidase activity thus transforming the predominant isoflavone glycosides to the aglycone forms as in our earlier study (Chapter 4.0). Each of the 4 bottles after the fermentation was then stored at various temperatures (-80°C, 4°C, 25°C, and 37°C) for 8 weeks. Aliquots of 50 mL were withdrawn aseptically at weekly intervals for 8 weeks and then stored immediately at -80°C for analysis of isoflavones. The frozen 50 mL aliquot was freeze-dried using a Dynavac® FD300 freeze drier (Rowville, Vic, Australia) for isoflavone extraction and analysis using reverse-phase high-performance liquid chromatography (HPLC).

5.2.5 Extraction of isoflavones for HPLC analysis

The extraction of isoflavones, including malonyl-, acetyl-, and β -Glycosides, and aglycones from fermented and non-fermented soymilk was performed in triplicate using a modified version of a

method described in section 4.2.6. A 1-g freeze-dried sample was added to 50 mL of methanol in a 150 mL round bottom flask and refluxed on a heating mantle for 1 h. The mixture was then filtered through a Whatman No. 1 filter paper into a 100 mL volumetric flask. The remaining dried soy matter was washed with the filtered portion and then refiltered into the same flask. A 5 mL aliquot was taken and after adding 60 μ L of internal standard (ISTD) flavone solution (10 mg/50 mL), the sample was dried under a stream of nitrogen using a Techne sample concentrator (Pearce Biotechnology Inc., Rockford, IL, USA). The resultant dried matter was then resuspended in 1 mL of 10 mM ammonium acetate buffer (containing 0.1% trifluoro-acetic acid) and methanol (50:50) solution and centrifuged ($14,000 \times g$ for 30 min) using an Eppendorf centrifuge (model 5415C; Crown Scientific Pty. Ltd., Vic, Australia), prior to transferring to HPLC vials.

5.2.6 Reversed-phase HPLC apparatus and reagents

Chromatographic analyses were carried out on a Hewlett Packard® 1100 series HPLC (Agilent Technologies, Forest Hill, Vic, Australia) with autosampler, quaternary pump, diode array ultraviolet (UV) visible detector, vacuum degasser, and thermostatically controlled column compartment as described in section 4.3.2. Alltech Alltima (Deerfield, IL, USA) HP C18HL (250 mm \times 4.6 mm internal diameter, 5 μ m) reversed-phase column attached to an Alltima HP C18HL (7.5 mm \times 4.6 mm internal diameter, 5 μ m) guard column was used to separate the isoflavone compounds. HPLC-grade methanol was purchased from Labscan Analytical Sciences (Bangkok, Thailand) and ammonium acetate from Sigma. All reagents used in isoflavone extraction and HPLC analyses were filtered through a 0.5 - μ m FH membrane (Millipore®, Bedford, MA, USA).

5.2.7 HPLC analysis of isoflavones

HPLC isocratic elution was used to isolate the isoflavones for detection and the mobile phase consisted of 100% methanol and 10 mM ammonium acetate buffer (50:50) containing 1 mL of trifluoro-acetic acid per litre of solvent mixture. This was set at a flow rate of 0.95 mL/min. Injection volumes of isoflavone standards and of the samples were set at 20 μ L throughout the run time of 30 min. A diode array UV-visible detector was set at dual wavelengths of 259 nm to detect the malonyl-, acetyl-, and β -glycosides, aglycones, and the flavone (ISTD), and 280 nm to detect equol. Single standards were prepared for peak identification. Malonyl- and acetyl-glycoside conjugates were quantified with respect to their β -glycoside equivalent response factors and corrected according to molecular weight. Isoflavone concentrations were calculated back to wet basis (mg isoflavones/ 100 mL soymilk).

5.2.8 Statistical analysis

The quantification of isoflavones and pH measurements were performed in triplicate on 2 trials. The data presented are means of 6 measurements and are presented as a mean \pm standard error of 6 analyses. To find the difference in isoflavone concentrations in soymilk during storage at different temperatures, means were analysed using one-way analysis of variance (ANOVA) and 99% confidence levels using Microsoft[®] Excel Statpro[®] as described by Albright *et al.* (1999). ANOVA data with a $P < 0.01$ was classified as statistically significant.

5.3 RESULTS AND DISCUSSION

5.3.1 Changes in the concentration of isoflavones during storage at different temperatures

Table 5.1 shows the concentration of isoflavones detected in fermented soymilk using *B.animalis ssp. lactis* Bb12 during 8 weeks storage at -80°C . There was no significant change in the concentration of glycoside glycitin and daidzin while the concentration of genistin significantly changed in the first week but appeared to be stable in the subsequent weeks of storage. As for the isoflavone aglycone isomers of glycitein and genistein, their concentrations did not significantly change ($P<0.01$) throughout the entire storage period while the concentration of daidzein changed significantly in the first week of storage but remained stable for subsequently. There was a significant ($P<0.01$) drop in the total isoflavone concentration during storage at -80°C . Although some isoflavone components remained significantly stable ($P<0.01$) during storage at -80°C , there was a slight decrease in their concentrations with time during storage. In a previous study (Chapter 3.0), we found that at peak β -glucosidase activity, maximum biotransformation of isoflavones occurred. The concentrations of isoflavones during peak enzyme activity during incubation were regarded as the initial concentration of the isoflavone isomers. During storage at -80°C , isoflavone glycosides of glycitin, daidzin and genistin reduced in their concentrations to 30.8%, 45.5% and 46.6%, respectively compared to the bioactive aglycone components of glycitein, daidzein and genistein whose concentrations only reduced to 62.4%, 79.7% and 85.2% of the initial concentrations, respectively. Therefore, it was observed that at -80°C , the isoflavone aglycones were more stable than that of their glycoside derivatives.

Table 5.2 shows the concentration of isoflavone isomers in fermented soymilk during storage at 4°C . Of the 6 isoflavone isomers quantified, only the isoflavone glycosides were not significantly ($P<0.01$) stable. All isoflavone aglycones of glycitein, daidzein and glycitein were, however,

significantly ($P < 0.01$) stable throughout the storage period. The isoflavone glycoside glycitein lost as much as 74.7% of its original concentration of 0.162 mg/100 mL of soymilk during 8 weeks of storage at 4°C. Daidzin and genistin concentrations reduced to 32.9% and 43.3% of the initial peak concentrations after 8 weeks, respectively at 4°C (Table 5.2), as compared to 30.8%, 45.8% and 46.6% at week 8, respectively for storage at -80°C (Table 5.1). Comparatively, it was observed that more glycoside losses occurred during storage at 4°C (Table 5.2) than at -80°C (Table 5.1). Glycitein, daidzein and genistein experienced losses in their concentrations of 32.8%, 15.3% and 12.4% at week 8, respectively during storage at 4°C (Table 5.2), compared to 37.6%, 20.3% and 14.8% at week 8, respectively, during storage at -80°C (Table 5.1). There were less isoflavone aglycone losses during storage at 4°C (Table 5.2) as compared to storage at -80°C (Table 5.1). However, glycitein incurred more losses compared to daidzein and genistein at both storage temperatures, implying that it is less stable than daidzein and genistein. Isoflavone glycosides incurred more losses compared to the aglycone constituents during storage at both temperatures.

Table 5.3 shows the concentration of isoflavones in fermented soymilk during storage at room temperature (25°C). Daidzein and genistein were significantly stable ($P < 0.01$) while glycitein was only stable after the first week of storage. The concentration of genistin reduced greatly after the first week of storage but remained relatively stable for the rest of the storage period while the other glycosides also reduced in concentration with storage time but the losses were not significant ($P < 0.01$). After 8 weeks of storage, the residual concentrations of the isoflavone aglycones glycitein, daidzein and genistein were 58.6%, 89% and 92.2%, respectively.

Table 5.4 shows the variations in the concentration of the isoflavones in fermented soymilk during storage at 37°C. The concentration of glycitein, daidzein and genistein at week 8 reduced

to 75.2%, 92.7% and 83.6%, respectively. On the other hand, the concentration of glycosides glycitin, daidzin and genistin substantially reduced to 30.2%, 57.2% and 5% respectively, during storage at 25°C (Table 5.3) compared to 62.3%, 74.8% and 13.8% during storage at 37°C (Table 5.4). Interestingly, it was observed that there was greater residual concentration of isoflavone glycosides at higher storage temperatures of 25°C (Table 5.3) and 37°C (Table 5.4), respectively as compared to storage at lower temperatures of -80°C (Table 5.1), and 4°C (Table 5.2). The storage temperature with the highest residual glycoside concentration at week 8 was 37°C (Table 5.4) with losses in glycitin and daidzin of only 37.7% and 25.2%, respectively. Our study reveals that glycosides are less stable (reduces more) than the bioactive isoflavone aglycones. The relatively higher residual concentration of the isoflavone β -glycosides at higher storage temperatures could be attributed to a comparatively reduced β -glucosidase activity as a result of the death of cells at later stages of storage. In our previous study on the stability of β -glucosidase in fermented soymilk (Chapter 3.0), we reported that the endogenous enzyme from probiotic *B.animalis ssp. lactis* Bb12 was more stable at lower storage temperature than at higher storage temperatures.

In general, aglycone genistein experienced least losses as compared to the other isoflavones during storage at -80°C (Table 5.1), at 4°C (Table 5.2) and at 25°C (Table 5.3). Daidzein on the other hand, experienced least losses during storage at 37°C (Table 5.4), compared to all other isoflavone constituents. There was only a 7.3% reduction in the concentration of daidzein as compared to 86.2% loss in the concentration of glycoside genistin in the same storage temperature at week 8. Isoflavone aglycones appeared to have experienced less losses as compared to their respective glycosides at all the four storage temperatures used in the study. Different mechanisms have been proposed for the degradation of soy isoflavones at different

temperatures. Xu *et al.* (2002) found that there was a temperature – dependent degradation pattern for soy isoflavones when heated above 135°C. They also observed that the glycosides tended to be even less stable in higher temperatures due to the co-current de-esterification of acetyl- and malonyl- derivatives to the corresponding underivatized glycosides. The aglycones, however, have been found to be more stable than glycosides during heating with glycitein and genistein degrading much faster compared to daidzein at temperatures above 135°C.

Of the 7 isoflavone isomers quantified over storage, equol was detected in trace amounts, the highest detectable concentration being at 0.004 mg/100 mL of soymilk. Equol was not detected until the third week storage at –80°C (Table 5.1), fifth week at 4°C (Table 5.2), while it took only 2 weeks to be detected during storage at 25°C (Table 5.3) and 37°C (Table 5.4). Equol is usually an exclusive product of intestinal bacterial metabolism of daidzein and its conjugates. According to plasma and urinary pharmacokinetic studies, only about 30 to 40% of the adult population is able to synthesise equol after ingesting soy foods or isoflavone supplements. The reason for these two distinct subpopulations is unclear at this point in time, but maybe due to the intestinal microbial composition and its influence on the intestinal enzyme systems involved in metabolic activity (Setchell *et al.* 2003).

Genistein was detected in highest concentration compared to daidzein and glycitein. The reason for higher concentration of genistein is due to its high level of corresponding Glycoside conjugates in the unfermented soy media. When these glycosides were hydrolysed by bacterial β -glucosidase, the result was a significant ($P<0.05$) decrease in their concentrations and a significant increase ($P<0.05$) in the concentration of isoflavone aglycones reaching as high as 3.185 mg/100 mL of soymilk (Table 4.5). The increment in the concentration of bioactive isoflavone aglycones through microbial β -glucosidases is, therefore, important step in enhancing

the potential clinical effectiveness of soy based foods (Chapter 4.0). We have previously shown that *B.animalis ssp. lactis* Bb12 possess β -glucosidase enzymes and show the enzyme activity in soymilk leading to isoflavone biotransformation (Chapter 3.0).

5.3.2 Kinetics of soy isoflavone degradation during storage

The ability to predict the impact of specific storage temperatures on isoflavone contents and composition is crucial to the optimisation of the concentration of bioactive isoflavone aglycones in fermented soymilk for guaranteeing potential health benefits. This requires obtaining reliable data on the kinetics of isoflavone degradation under the likely storage conditions depending on the product characteristics. The kinetics of the individual isoflavone isomers was determined by monitoring their concentrations in soymilk samples stored at various temperatures (-80°C, 4°C, 25°C and at 37°C). Eissen *et al.* (2003) determined the loss of isoflavone Glycoside genistin in soymilk and found that its decrease appeared to be of first order of kinetics. In Table 5.5, we assumed a first order of kinetics to determine degradation constants for the individual isoflavone glycosides and aglycones at all the storage temperatures for up to 8 weeks (56 d). Using the concentrations of Glycosides and aglycones during peak β -glucosidase activity (Table 3.2) at incubation as initial concentrations (C_0), the kinetic degradation constants were calculated based on the weekly concentrations of the isoflavone isomers. The initial isoflavone concentrations used for calculating the rate of loss are shown in Table 5.1 to 5.4 and the first order of kinetics models are shown in Figures 5.1 to 5.4. In first order of kinetics, the rate of reaction is proportional to the concentration of a single reactant raised to the first power (Keusch, 2005). The decrease in the concentration of isoflavone (C) over time can be written as:

$$v = -\frac{d[C]}{dt} = k[C] \dots \dots \dots (1)$$

$$-\frac{d[C]}{C} = kdt \dots\dots\dots(2)$$

Equation (2) represents the differential form of the rate law. Integration of this equation and determination of the integration constant B produces the corresponding integrated law.

Intergrating equation (2) yields

$$\ln[C] = -kt + B \dots\dots\dots(3)$$

The constant of integration B can be evaluated by using boundary conditions. When

$$t = 0, [C] = [C]_o . [C]_o \text{ is the original concentration of C .}$$

Substituting into equation (3) gives:

$$\ln[C]_o = -k(0) + C \dots\dots\dots(4)$$

Therefore the value of the constant of integration is:

$$B = \ln[C]_o \dots\dots\dots(5)$$

Substituting (5) into (4) leads to:

$$\ln \frac{[C]}{[C]_o} = -kt \dots\dots\dots(6)$$

Plotting $\ln[C]$ or $\frac{[C]}{[C]_o}$ against time creates a straight line with slope $-k$;

where C is the concentration of the isoflavone isomer during storage at a specified temperature after a specific period of time, and C_o , is the initial concentration of the isomer at peak β -glucosidase activity. The plot is usually linear up to about 90% conversions (Keusch, 2005).

As shown in Table 5.5, isoflavone glycosides of glycitin, daidzin and genistin showed greater degradation constants compared to the aglycone components. The degradation constant was the calculated loss of the isoflavones per day in the product at the specific storage temperature. A higher degradation constant implies higher daily losses and vice versa for low constants. In

fermented soymilk with *B.animalis ssp. lactis Bb12*, the glycoside genistin showed the highest daily loss of 0.0545 mg/100 mL per day during storage at 25°C, while genistein had the least calculated daily loss of 0.0017 mg/100 mL per day during storage at 25°C and 37°C. Daidzein had a very similar degradation pattern to that of genistein despite having different concentrations in the soymilk. The almost superimposed lines in Figures 5.1 to 5.4 best exemplify the similarity of degradation of the two aglycones in soymilk during storage. Glycitein, on the other hand, was less stable than daidzein and genistein as indicated by the higher degradation constants at all storage temperatures. The occurrence of methoxyl (-OCH₃) group in glycitein could possibly be the difference for the comparatively higher degradation as compared to that of daidzein and genistein. Glycitein possesses a methoxyl group at position 6 of its A-ring, whereas genistein and daidzein have hydroxyl (-OH) groups at different positions other than position 6 but both without a methoxyl group.

Genistin losses in fermented soymilk were higher at higher storage temperatures (25°C and 37°C) at 0.0545 and 0.0525 mg/100 mL of soymilk per day, respectively, as compared to lower storage temperatures of -80°C and 4°C, whose degradation constants were 0.0182 and 0.0328 mg/100 mL of soymilk per day, respectively. A similar result was also obtained by Eissen *et al.* (2003), in which they observed a higher degradation constant for genistin in soymilk during a 6-month storage experiments at ambient conditions. Whereas different isoflavone isomers showed different degradation constants at different storage temperatures, there was not so much difference in the degradation constants of the sum total of isoflavones isomers in the soymilk. Storage at 37°C gave the least total isoflavone degradation constant of 0.002 mg/100 mL of soymilk per day while -80°C, 4°C and 25°C gave degradation constants of 0.0053 mg/100 mL, 0.0050 mg/100 mL and 0.0047 mg/100 mL of soymilk per day, respectively. These losses may

have largely been due to the co-current de-esterification of acetyl and malonyl derivatives to the underivatized glycosides. Davies *et al.* (1998) reported another potential route for isoflavone degradation in composite solutions, suggesting that isoflavones could react in Maillard type reactions. It is not yet known as to at what temperature Maillard type of reaction occurs in a given soy media. It can, however, be deduced that part of the reason for the very low degradation of isoflavones at lower temperatures is the reduced chance of, or even non existence of Maillard type of reaction.

From the data, however, it is evident that the bioactive isoflavone aglycones degrade much less as compared to the Glycoside components during storage in fermented soymilk. This is important in the design of storage conditions of the product to guarantee minimal losses of the bioactive components, which could consequently confer health benefits to the consumer. Glycitein degradation appeared to be linearly dependent on the storage temperature with higher temperatures causing higher losses and lower storage temperatures causing minimal losses. This did not appear to be the case with daidzein and genistein, although the difference in losses between the highest and the lowest storage temperatures was negligible (<0.0015 mg/100 mL of soymilk). How various factors interact to determine individual isoflavone isomer degradation is still not fully understood. Thus a linear model such as first order kinetic, though important in determining the rate of degradation, is inadequate for use in explaining the different degradation mechanisms of different isoflavone isomers. According to Shimoni (2004), beyond the first order degradation kinetics, there is a change in the isoflavone profile according to processing and storage conditions; this is yet to be fully comprehended. Even though this mechanism cannot be fully explained, the rate of loss of isoflavones can still be used as a safe guide in determining storage conditions for special products with the bioactive components. Our previous study (Chapter 3.0) showed that 4°C was a much better storage temperature for fermented soymilk with

β -glucosidase producing probiotics due to better stability of the enzyme activity. The enzyme being responsible for the significant increase ($P < 0.05$) in the aglycone component played some role in the stability of these bioactive components. In the current study, storage at 4°C yielded degradation constants for genistein, daidzein and glycitein as 0.0021, 0.0022 and 0.0193 mg/100 mL of soymilk per day, respectively. Although the rate of degradation appeared to be slow for soy isoflavones under these storage conditions, the fact that isoflavones aglycones are key bioactive compounds may be important in terms of defining "end of shelf life". It is therefore reasonable to infer that storage at 4°C did guarantee minimal degradation of bioactive isoflavone aglycones.

5.4 CONCLUSIONS

Both the isoflavone glycosides and aglycones were found to be very stable in all the storage temperatures used. Using a first order of kinetics model to determine the degradation constants in such storage conditions, a much better understanding of the individual isoflavones losses was obtained. Bioactive aglycones had much smaller degradation constants as compared to the glycoside isoflavone components. Of the aglycones, daidzein and genistein had a similar degradation pattern in all the storage temperatures with almost equal degradation constants, despite their difference in concentration and molecular masses. Glycitein, on the other hand, had higher degradation constants compared to those of daidzein and genistein. The first order of kinetics model while important in assessing the rate of loss at storage temperatures was insufficient in understanding the mechanisms involved in the losses. In general, the storage temperature of 4°C was found to be the most ideal temperature for the product in order to guarantee minimal degradation of bioactive isoflavone aglycones.

Table 5.1 Concentration of isoflavones (mg/ 100 mL) in fermented soymilk using *B.animalis ssp. lactis Bb12* during storage at -80°C

Isoflavone isomers	^A Initial concentrations	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Glycitin	$0.162^a \pm 0.02$	$0.072^a \pm 0.01$	$0.075^a \pm 0.04$	$0.050^a \pm 0.02$	$0.061^a \pm 0.04$	$0.029^a \pm 0.005$	$0.046^a \pm 0.03$	$0.028^a \pm 0.03$	$0.050^a \pm 0.03$
Daidzin	$0.155^a \pm 0.03$	$0.094^a \pm 0.03$	$0.051^a \pm 0.02$	$0.042^a \pm 0.003$	$0.082^a \pm 0.01$	$0.056^a \pm 0.01$	$0.070^a \pm 0.02$	$0.072^a \pm 0.02$	$0.071^a \pm 0.03$
Genistin	$0.283^a \pm 0.05$	$0.143^{ab} \pm 0.01$	$0.084^b \pm 0.02$	$0.150^{ab} \pm 0.02$	$0.147^{ab} \pm 0.01$	$0.164^{ab} \pm 0.012$	$0.140^{ab} \pm 0.03$	$0.133^b \pm 0.02$	$0.132^b \pm 0.03$
Glycitein	$0.210^a \pm 0.01$	$0.116^a \pm 0.03$	$0.126^a \pm 0.02$	$0.143^a \pm 0.04$	$0.129^a \pm 0.03$	$0.100^a \pm 0.01$	$0.101^a \pm 0.03$	$0.126^a \pm 0.02$	$0.131^a \pm 0.02$
Daidzein	$0.997^a \pm 0.05$	$0.881^{ab} \pm 0.01$	$0.949^{ab} \pm 0.03$	$0.905^{ab} \pm 0.01$	$0.914^{ab} \pm 0.01$	$0.865^{ab} \pm 0.01$	$0.895^{ab} \pm 0.01$	$0.916^{ab} \pm 0.05$	$0.795^{ab} \pm 0.03$
Genistein	$2.876^a \pm 0.11$	$2.559^a \pm 0.08$	$2.638^a \pm 0.09$	$2.616^a \pm 0.08$	$2.540^a \pm 0.09$	$2.592^a \pm 0.04$	$2.551^a \pm 0.03$	$2.594^a \pm 0.07$	$2.451^a \pm 0.09$
Equol	ND	ND	ND	$0.001^a \pm 0.00$	$0.001^a \pm 0.00$	$0.004^a \pm 0.002$	$0.003^a \pm 0.002$	$0.001^a \pm 0.00$	$0.003^a \pm 0.00$
Total Isoflavones	$4.683^a \pm 0.03$	$3.864^b \pm 0.03$	$3.923^b \pm 0.03$	$3.907^b \pm 0.03$	$3.874^b \pm 0.04$	$3.809^b \pm 0.01$	$3.806^b \pm 0.04$	$3.870^b \pm 0.001$	$3.634^b \pm 0.12$

Results expressed as mean \pm standard error in mg/ 100 mL of fermented soymilk (n = 6). Statistical analysis by means of one-way ANOVA

^{a,b}Means in the same row with different lowercase superscripts are significantly different ($P < 0.01$)

^AInitial concentration - is the concentration of isoflavones during peak β -glucosidase activity resulting from 24 h incubation at 37°C

Table 5.2 Concentration of isoflavones (mg/100 mL) in fermented soymilk using *B.animalis ssp. lactis Bb12* during storage at 4°C

Isoflavone isomers	^A Initial concentrations	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Glycitin	0.162 ^a ± 0.02	0.071 ^a ± 0.05	0.043 ^a ± 0.01	0.046 ^a ± 0.02	0.045 ^a ± 0.02	0.031 ^a ± 0.001	0.085 ^a ± 0.05	0.041 ^a ± 0.01	0.048 ^a ± 0.01
Daidzin	0.155 ^a ± 0.03	0.122 ^{ab} ± 0.03	0.051 ^{ac} ± 0.01	0.033 ^{bc} ± 0.002	0.029 ^{bc} ± 0.004	0.028 ^{bc} ± 0.01	0.065 ^{ab} ± 0.02	0.048 ^{ab} ± 0.01	0.051 ^{ab} ± 0.01
Genistin	0.283 ^a ± 0.05	0.134 ^b ± 0.03	0.130 ^b ± 0.01	0.126 ^b ± 0.02	0.093 ^b ± 0.01	0.073 ^b ± 0.01	0.079 ^b ± 0.02	0.063 ^b ± 0.01	0.058 ^b ± 0.01
Glycitein	0.210 ^a ± 0.01	0.134 ^a ± 0.06	0.076 ^a ± 0.03	0.102 ^a ± 0.002	0.072 ^a ± 0.02	0.104 ^a ± 0.01	0.095 ^a ± 0.03	0.089 ^a ± 0.01	0.090 ^a ± 0.02
Daidzein	0.997 ^a ± 0.05	0.960 ^a ± 0.06	0.987 ^a ± 0.05	0.895 ^a ± 0.005	0.903 ^a ± 0.01	0.925 ^a ± 0.01	0.884 ^a ± 0.02	0.844 ^a ± 0.03	0.984 ^a ± 0.03
Genistein	2.876 ^a ± 0.11	2.436 ^a ± 0.15	2.601 ^a ± 0.07	2.689 ^a ± 0.05	2.669 ^a ± 0.04	2.752 ^a ± 0.03	2.574 ^a ± 0.08	2.784 ^a ± 0.04	2.518 ^a ± 0.14
Equol	ND	ND	ND	ND	ND	0.001 ^a ± 0.00	0.003 ^a ± 0.000	0.001 ^a ± 0.00	0.000 ^a ± 0.00
Total Isoflavones	4.683 ^a ± 0.03	3.858 ^b ± 0.02	3.888 ^b ± 0.01	3.891 ^b ± 0.03	3.811 ^b ± 0.04	3.914 ^b ± 0.02	3.784 ^b ± 0.03	3.871 ^b ± 0.02	3.748 ^b ± 0.07

Results expressed as mean ± standard error in mg/100 mL of fermented soymilk (n = 6). Statistical analysis by means of one-way ANOVA

^{a,b,c}Means in the same row with different lowercase superscripts are significantly different (P < 0.01)

^AInitial concentration - is the concentration of isoflavones during peak β-glucosidase activity resulting from 24 h incubation at 37°C

Table 5.3 Concentration of isoflavones (mg/100 mL) in fermented soymilk using *B.animalis ssp. lactis Bb12* during storage at 25°C

Isoflavone isomers	^A Initial concentrations	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Glycitin	0.162 ^a ± 0.02	0.043 ^a ± 0.02	0.041 ^a ± 0.02	0.045 ^a ± 0.03	0.075 ^a ± 0.03	0.065 ^a ± 0.02	0.105 ^a ± 0.03	0.056 ^a ± 0.004	0.049 ^a ± 0.01
Daidzin	0.155 ^a ± 0.03	0.036 ^a ± 0.004	0.028 ^a ± 0.006	0.034 ^a ± 0.01	0.046 ^a ± 0.01	0.071 ^a ± 0.02	0.073 ^a ± 0.03	0.060 ^a ± 0.03	0.089 ^a ± 0.05
Genistin	0.283 ^a ± 0.05	0.059 ^b ± 0.005	0.024 ^b ± 0.003	0.014 ^b ± 0.003	0.025 ^b ± 0.005	0.047 ^b ± 0.01	0.019 ^b ± 0.01	0.044 ^b ± 0.03	0.014 ^b ± 0.003
Glycitein	0.210 ^a ± 0.01	0.086 ^b ± 0.01	0.100 ^{ab} ± 0.01	0.115 ^{ab} ± 0.01	0.073 ^b ± 0.02	0.118 ^{ab} ± 0.01	0.129 ^{ab} ± 0.03	0.151 ^{ab} ± 0.03	0.123 ^{ab} ± 0.02
Daidzein	0.997 ^a ± 0.05	0.949 ^a ± 0.05	0.952 ^a ± 0.02	0.966 ^a ± 0.01	0.977 ^a ± 0.01	0.918 ^a ± 0.01	0.925 ^a ± 0.02	0.923 ^a ± 0.03	0.887 ^a ± 0.01
Genistein	2.876 ^a ± 0.11	2.746 ^a ± 0.07	2.766 ^a ± 0.03	2.745 ^a ± 0.04	2.718 ^a ± 0.04	2.718 ^a ± 0.04	2.691 ^a ± 0.05	2.661 ^a ± 0.09	2.653 ^a ± 0.05
Equol	ND	ND	0.001 ^a ± 0.00	0.001 ^a ± 0.00	ND	0.003 ^a ± 0.00	0.002 ^a ± 0.000	0.002 ^a ± 0.00	0.001 ^a ± 0.00
Total Isoflavones	4.683 ^a ± 0.03	3.920 ^b ± 0.03	3.913 ^b ± 0.01	3.924 ^b ± 0.03	3.914 ^b ± 0.03	3.914 ^b ± 0.02	3.911 ^b ± 0.01	3.896 ^b ± 0.04	3.817 ^b ± 0.01

Results expressed as mean ± standard error in mg/100 mL of fermented soymilk (n = 6). Statistical analysis by means of one-way ANOVA

^{a,b,c}Means in the same row with different lowercase superscripts are significantly different (P < 0.01)

^AInitial concentration - is the concentration of isoflavones during peak β-glucosidase activity resulting from 24 h incubation at 37°C

Table 5.4 Concentration of isoflavones (mg/100 mL) in fermented soymilk using *B.animalis ssp. lactis* Bb12 during storage at 37°C

Isoflavone isomers	^A Initial concentrations	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Glycitin	0.162 ^a ± 0.02	0.024 ^b ± 0.003	0.049 ^b ± 0.01	0.078 ^{ab} ± 0.01	0.068 ^{ab} ± 0.003	0.105 ^{ab} ± 0.003	0.108 ^{ab} ± 0.01	0.108 ^{ab} ± 0.003	0.101 ^{ab} ± 0.003
Daidzin	0.155 ^a ± 0.03	0.030 ^a ± 0.008	0.060 ^a ± 0.02	0.098 ^a ± 0.04	0.135 ^a ± 0.06	0.104 ^a ± 0.07	0.140 ^a ± 0.05	0.101 ^a ± 0.03	0.116 ^a ± 0.05
Genistin	0.283 ^a ± 0.05	0.043 ^b ± 0.02	0.023 ^b ± 0.003	0.019 ^b ± 0.006	0.022 ^b ± 0.006	0.031 ^b ± 0.01	0.036 ^b ± 0.01	0.063 ^b ± 0.02	0.039 ^b ± 0.006
Glycitein	0.210 ^a ± 0.01	0.094 ^a ± 0.02	0.088 ^a ± 0.01	0.080 ^a ± 0.03	0.291 ^a ± 0.02	0.136 ^a ± 0.05	0.093 ^a ± 0.02	0.051 ^a ± 0.02	0.158 ^a ± 0.04
Daidzein	0.997 ^a ± 0.05	0.955 ^a ± 0.03	0.933 ^a ± 0.02	0.932 ^a ± 0.01	0.831 ^a ± 0.03	0.868 ^a ± 0.03	0.872 ^a ± 0.02	0.885 ^a ± 0.01	0.924 ^a ± 0.01
Genistein	2.876 ^a ± 0.11	2.715 ^a ± 0.05	2.804 ^a ± 0.03	2.740 ^a ± 0.05	2.576 ^a ± 0.11	2.603 ^a ± 0.05	2.606 ^a ± 0.01	2.624 ^a ± 0.04	2.403 ^a ± 0.017
Equol	ND	ND	0.001 ^{ab} ± 0.00	0.001 ^{ab} ± 0.00	ND	0.003 ^a ± 0.00	0.004 ^a ± 0.001	0.001 ^{ab} ± 0.00	0.001 ^{ab} ± 0.00
Total Isoflavones	4.683 ^a ± 0.03	3.860 ^b ± 0.03	3.958 ^b ± 0.01	3.948 ^b ± 0.03	3.924 ^b ± 0.01	3.849 ^b ± 0.04	3.860 ^b ± 0.01	3.826 ^b ± 0.02	3.755 ^b ± 0.03

Results expressed as mean ± standard error in mg/100 mL of fermented soymilk (n = 6). Statistical analysis by means of one-way ANOVA

^{a,b}Means in the same row with different lowercase superscripts are significantly different (P < 0.01)

^AInitial concentration - is the concentration of isoflavones during peak β-glucosidase activity resulting from 24 h incubation at 37°C

Table 5.5 Kinetic parameters for degradation of soy isoflavones in fermented soymilk using *B.animalis ssp. lactis* Bb12 during storage

Isoflavone compound	Storage temperature (°C)	¹ Rate constant = $\kappa \times 10^2/\text{day}^{-1}$
Glycitin	-80	3.33
	4	3.07
	25	2.46
	37	1.44
Daidzin	-80	2.15
	4	2.65
	25	2.36
	37	0.94
Genistin	-80	1.82
	4	3.28
	25	5.45
	37	5.25
Glycitein	-80	1.41
	4	1.93
	25	1.85
	37	1.98
Daidzein	-80	0.32
	4	0.22
	25	0.19
	37	0.26
Genistein	-80	0.30
	4	0.21
	25	0.17
	37	0.17
Total Isoflavones	-80	0.53
	4	0.50
	25	0.47
	37	0.40

¹Rate constant (κ) – is the amount of isoflavone losses in mg/100 mL of soymilk per day (1/d) at the specific storage temperature

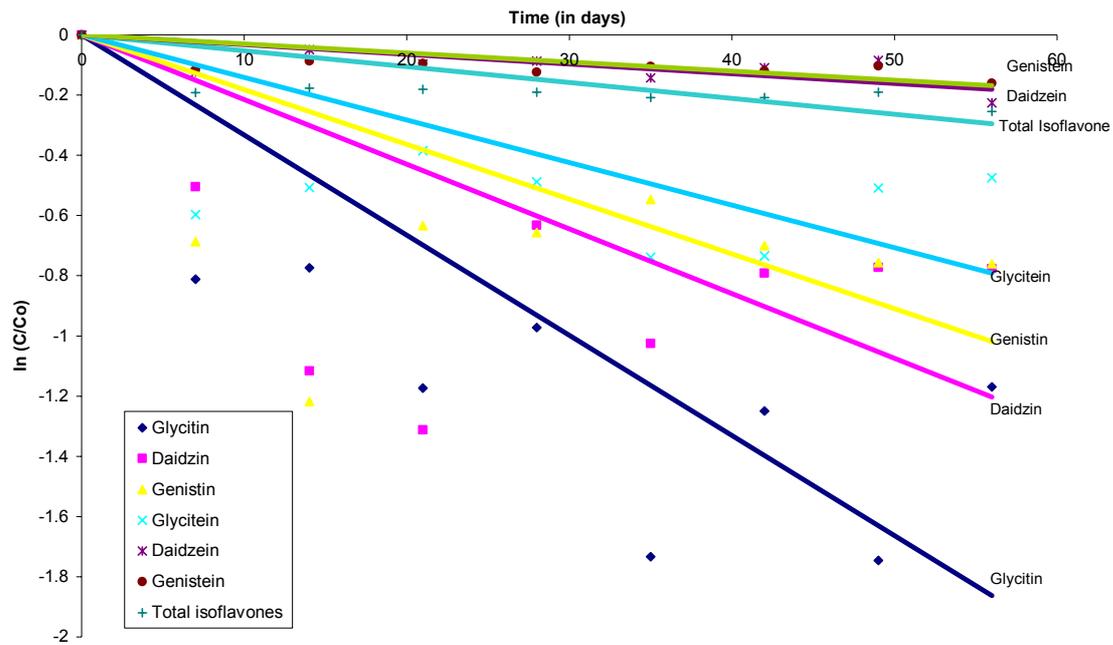


Figure 5.1 Isoflavone degradation in fermented soymilk during storage at -80°C . $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone isomer to its initial concentration at the specified storage temperature

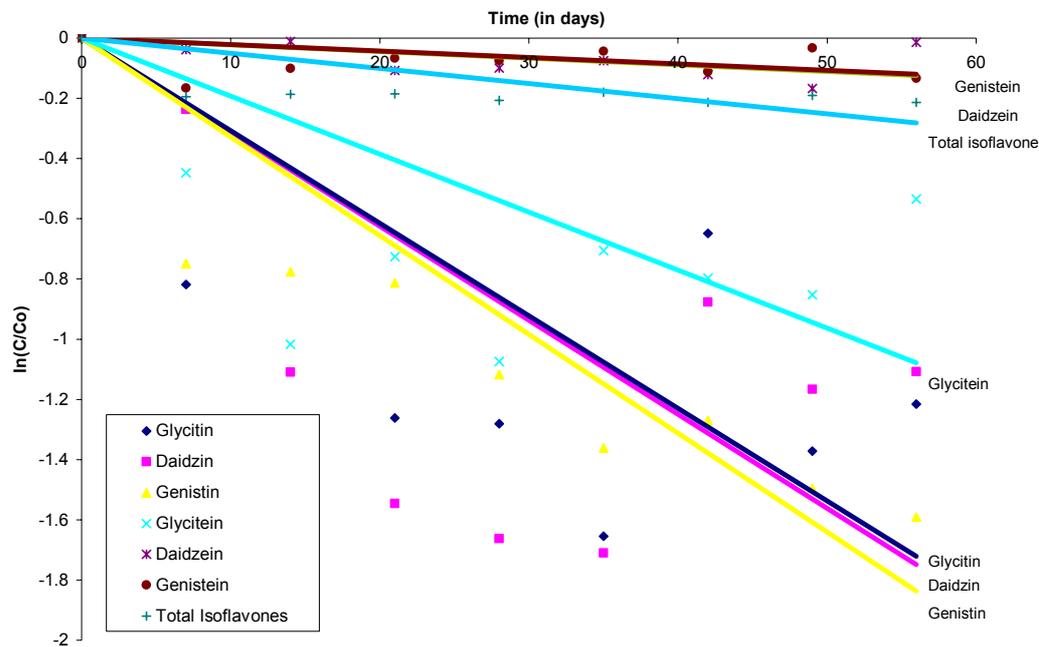


Figure 5.2 Isoflavone degradation in fermented soymilk during storage at 4°C. $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone isomer to its initial concentration at the specified storage temperature

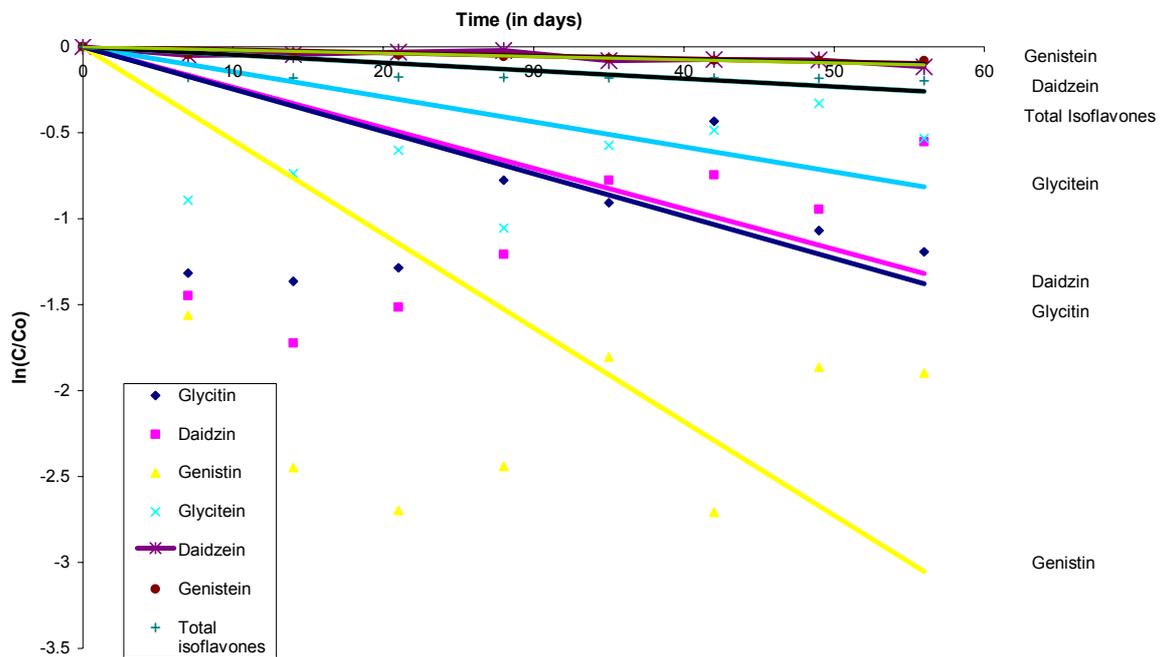


Figure 5.3 Isoflavone degradation in fermented soymilk during storage at 25°C. $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone isomer to its initial concentration at the specified storage temperature

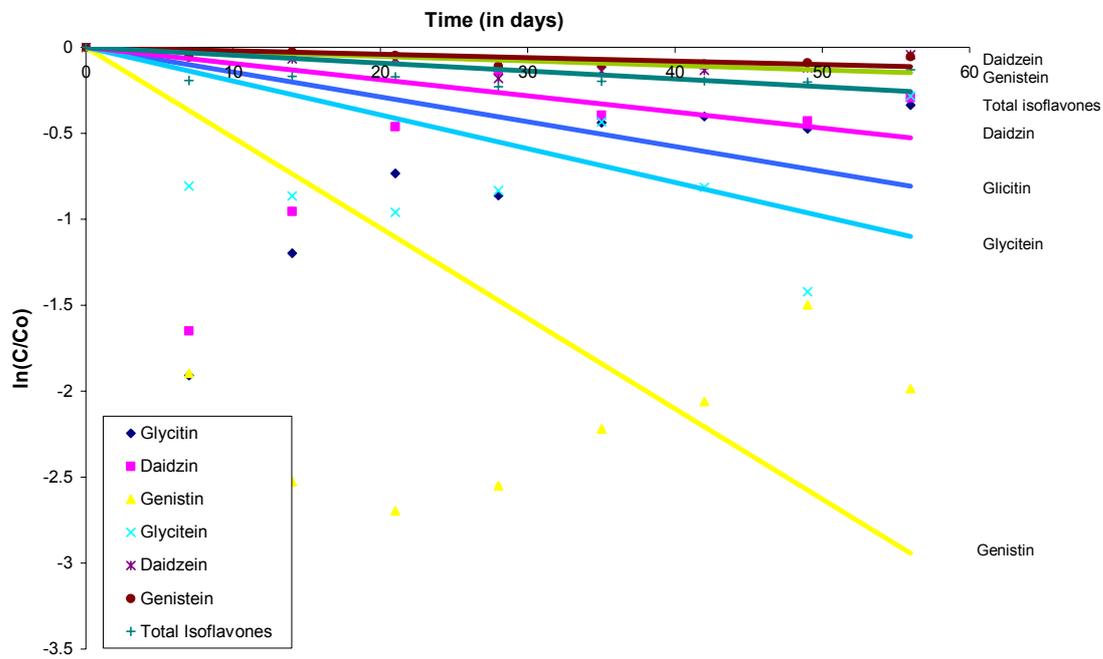


Figure 5.4 Isoflavone degradation in fermented soymilk during storage at 37°C. $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone isomer to its initial concentration at the specified storage temperature

6.0 Isoflavone phytoestrogen degradation in fermented soymilk with selected β -glucosidase producing *Lactobacillus acidophilus* strains during storage at different temperatures

6.1 INTRODUCTION

The ability to predict the impact of specific processing and storage conditions on isoflavones content and composition is crucial for optimization of processes. This requires obtaining reliable data on the kinetics of their degradation under various processing and storage conditions. Isoflavones are intrinsic plant compounds having a 1, 2 – diarylpropane structure. To date over 230 members of isoflavones have been identified (Cassidy *et al.* 2000). The aglycone structures of daidzein, genistein and glycitein, are the parent isoflavones which are conjugated to form malonyl-, acetyl- and β -glycoside configurations. The aglycons are structurally similar to the mammalian oestrogen oestradiol-17 β and therefore mimic some of the function of estradiol in the human body (Setchell & Cassidy, 1999). The isoflavones, daidzein and genistein for example, share identical structures except for an additional group on the A ring of genistein but genistein may have up to five-or six-fold greater oestrogenic activity (Cassidy *et al.*2000).

Lactobacillus acidophilus is an important component of probiotic microorganisms which benefit the host when viable cells are ingested (Shah, 2000). Thus the incorporation of β -glucosidase producing *L. acidophilus* strains in soymilk may lead to a combination of benefits as probiotics as well as that from transformation of isoflavone glycosides to bioactive isoflavone aglycones (Chapter 3.0). Moreover, their hydrolysing ability can influence the stability of isoflavones in soymilk during fermentation and storage.

Studies have shown that the overall content of the isoflavones in soy protein isolates and corn mixtures decreased when subjected to high temperatures at 110°C, 130°C, and 150°C (Mahungu

et al. 1999). However, most of these studies were limited to total isoflavone losses during processing at higher temperatures. Only Ungar *et al.* (2003) have demonstrated that genistein and daidzein solutions at pH 9.0 and 7.0 incubated at 70°C to 90°C apparently degrades following a first order kinetic model. To date little data are available on the kinetics of isoflavone degradation. Such data are crucial for the assessment of the health potential and clinical efficacy of soy based foods. To the best of our knowledge, there is no reported data having assessed the degradation of individual isoflavones in fermented soymilk during storage. It is important that once the biological activity of a soy-based product has been improved through fermentation, the stability of bioactive components be maintained via an optimum storage temperature in order to maintain adequate levels that would confer health benefits to the consumer. Hence, the objectives of this study were to examine the stability of isoflavone glycosides and aglycones in fermented soymilk during storage at various temperatures (-80°C, 4°C, 25°C and 37°C).

6.2 MATERIALS AND METHODS

6.2.1 Microorganisms

Pure culture of *L. acidophilus* 4461 was obtained from the Victoria University Culture Collection (Werribee, Vic., Australia) and *L. acidophilus* ATCC 4962 and *L. acidophilus* ATCC 33200 were from the Australian Starter Culture Research Centre (Werribee, Vic, Australia). The purity of the culture was checked and stored as described in section 3.2.1.

6.2.2 Bacterial growth media

Rehydrated de Mann Rogosa Sharpe (MRS) broth (pH adjusted to 6.7 using 5 M sodium hydroxide) (De Mann *et al.*1960) was prepared according to the method described in section 4.2.2.

6.2.3 Soymilk manufacture

Soy protein isolate (SPI; SUPRO 590), supplied by The Solae Co. (Chatswood, NSW, Australia), was used in the production of soymilk at 4% of ultra-pure distilled water as described in section 3.2.2. After reconstitution, the soymilk was dispensed into twelve glass bottles at 400 mL each, then autoclaved at 121°C for 15 min. After cooling to room temperature, the pH was adjusted in a laminar flow to 6.7 using 5 M sodium hydroxide.

6.2.4 Fermentation of soymilk with *L. acidophilus* 4461, *L. acidophilus* ATCC 4962 and *L. acidophilus* ATCC 33200

Activation of the microorganisms in MRS broth were conducted at 37°C for 20 h successively 3 times followed by a fourth activation in sterile soymilk. An inoculum level of 5% (v/v) and the incubation at 37°C for 20 h were used for activation. For storage studies, 24 bottles each containing 400 mL of sterile soymilk were inoculated (in duplicate) with the active culture (5% v/v), and incubated at 37°C for 24 h to achieve peak biotransformation of the predominant isoflavone Glycosides via β -glucosidase activity to the aglycone forms as reported in our earlier study (Chapter 4.0). After fermentation, 3 bottles (in duplicate) containing each microorganism were then stored at 4 different temperatures (-80°C, 4°C, 25°C, and 37°C) for 8 weeks. Fifty millilitre aliquots were withdrawn aseptically at weekly intervals and stored immediately at -80°C for analysis of isoflavones. The frozen aliquots were freeze-dried using a Dynavac® FD300 freeze drier (Rowville, Vic, Australia) for isoflavone extraction and analysis using reverse-phase high-performance liquid chromatography (HPLC).

6.2.5 Extraction of isoflavones from freeze dried samples

Solvent HPLC grade methanol was used in the extraction of isoflavones including malonyl-, acetyl-, and β -glycosides, and aglycones from fermented and non-fermented soymilk and was

performed in triplicate as mentioned in section 4.2.6. Briefly, a 1-g freeze-dried sample was added to 50 mL of methanol in a 150 mL round bottom flask and refluxed on a heating mantle for 1 h. The mixture was then filtered through a Whatman No. 1 filter paper into a 100 mL volumetric flask. The remaining dried soy matter was washed with the filtered portion and then refiltered into the same flask. A 5 mL aliquot was taken and after adding 50 μ L of internal standard (ISTD) flavone solution (10 mg/50 mL), the sample was dried under a stream of nitrogen using a Techne sample concentrator (Pearce Biotechnology Inc., Rockford, IL, USA). The resultant dried matter was then resuspended in 1 mL of 10 mM ammonium acetate buffer (containing 0.1% trifluoro-acetic acid) and methanol (50:50) solution and centrifuged ($14,000 \times g$ for 30 min) using an Eppendorf centrifuge (model 5415C; Crown Scientific Pty. Ltd., Vic, Australia), prior to transferring to HPLC vials.

6.2.6 Reversed-phase HPLC apparatus and reagents

Chromatographic runs were carried out on a Hewlett Packard® 1100 series HPLC (Agilent Technologies, Forest Hill, Vic, Australia) with autosampler, quaternary pump, diode array ultraviolet (UV) visible detector, vacuum degasser, and thermostatically controlled column compartment. Alltech Alltima (Deerfield, IL, USA) HP C₁₈HL (250 mm \times 4.6 mm internal diameter, 5 μ m) reversed-phase C₁₈ column attached to an Alltima HP C₁₈HL guard column (7.5 mm \times 4.6 mm internal diameter, 5 μ m) was used to separate the isoflavone compounds as mentioned in section 4.3.2. HPLC-grade methanol, trifluoro-acetic acid, absolute ethanol, and ammonium acetate from Sigma and all the isoflavone standards were obtained from sources mentioned in sections 4.2.8. All reagents used in isoflavone extraction and HPLC analyses were filtered through a 0.5 μ m FH membrane (Millipore®, Bedford, MA, USA).

6.2.7 HPLC analysis of isoflavones

HPLC isocratic elution was used to isolate the isoflavones for detection and the mobile phase consisted of 100% methanol and 10 mM ammonium acetate buffer (50:50) containing 1 mL of trifluoro-acetic acid per litre of solvent mixture. This was set at a flow rate of 0.95 mL/min according to our method in Chapter 5.0 (section 5.2.8). Injection volumes of isoflavone standards and of the samples, HPLC run time, column specifications and guard column and its specifications were according to our method outlined in chapter 5.0 (section 5.2.8). A diode array UV-visible detector was set at dual wavelengths of 259 nm to detect malonyl-, acetyl-, and β -glycosides, aglycones, and flavone (ISTD), and 280 nm to detect equol as mentioned in section 5.2.8. Single standards were prepared for peak identification. Isoflavone concentrations were calculated back to wet basis (ng/ μ L of soymilk).

6.2.8 Isoflavone identification

Retention times and UV absorption patterns of pure isoflavone standards were used to identify isoflavones in samples following extraction and HPLC analysis. The isoflavone standards including genistin, daidzin, glycitin, genistein, daidzein, and glycitein were detected at 259 nm, while equol was detected at 280 nm.

6.2.9 Standard solutions and calibration curves

Stock solutions for the different isoflavone standards were prepared by dissolving 1 mg of the crystalline pure compounds in 100% methanol. Each solution was then serially diluted with methanol to 5 working solutions of different standard concentrations in order to prepare a standard curve. Aliquots of working solutions were then analysed by HPLC, and the

concentration for each standard solution was calculated. These were used to calculate the concentrations of isoflavones present in freeze dried fermented soymilk.

6.2.10 Statistical analysis of data

The quantification of isoflavones was performed in triplicate on 2 trials and repeated. The data are means of 6 measurements and are presented as a mean \pm standard error of 12 analyses. To find the difference in isoflavone concentrations in soymilk during storage at different temperatures, means were analysed using one-way analysis of variance (ANOVA) and 99% confidence levels using Microsoft[®] Excel Statpro[®] as described by Albright *et al.* (1999). ANOVA data with a $P < 0.01$ was classified as statistically significant.

6.2.11 First order kinetics of isoflavone degradation

Based on these previous studies, first order kinetic model was used to study the degradation constants of isoflavones in fermented soymilk with *Lactobacillus* strains during storage at the four different storage temperatures. In first order of kinetics, the rate of reaction is proportional to the concentration of a single reactant raised to the first power (Keusch, 2005). The decrease in the concentration of isoflavone (C) over time can be written as:

$$v = -\frac{d[C]}{dt} = k[C] \dots \dots \dots (1)$$

$$-\frac{d[C]}{C} = kdt \dots \dots \dots (2)$$

Equation (2) represents the differential form of the rate law. Integration of this equation and determination of the integration constant B produces the corresponding integrated law.

Intergrating equation (2) yields:

$$\ln[C] = -kt + B \dots \dots \dots (3)$$

The constant of integration B can be evaluated by using boundary conditions. When

$$t = 0, [C] = [C]_0. [C]_0 \text{ is the original concentration.}$$

Substituting into equation (3) gives:

$$\ln[C]_0 = -k(0) + C \dots\dots\dots (4)$$

Therefore the value of the constant of integration is:

$$B = \ln[C]_0 \dots\dots\dots (5)$$

Substituting (5) into (4) leads to:

$$\ln \frac{[C]}{[C]_0} = -kt \dots\dots\dots (6)$$

Plotting $\ln[C]$ or $\frac{[C]}{[C]_0}$ against time creates a straight line with slope $-k$;

where C is the concentration of the isoflavone isomer during storage at a specified temperature after a specific time period and C_0 is the initial concentration of the isomer at peak β -glucosidase activity. The plot is usually linear up to about 90% conversions (Keusch, 2005).

6.3 RESULTS AND DISCUSSION

6.3.1 Changes in the concentration of isoflavones in fermented soymilk during storage

The initial isoflavone concentrations and changes in isoflavone compounds due to fermentation by *L. acidophilus* during storage at 4 different temperatures are shown in Table 6.1. Highest concentrations of isoflavone aglycones have been found to correspond to peak enzyme activity in fermented soymilk and the extent of hydrolysis was dependent on the hydrolytic potential of the endogenous β -glucosidase from each micro-organism (Chapter 4.0). Genistein had the highest concentrations followed by daidzein and then glycitein, while there was a decrease in glycitin, daidzin and genistin. Equol was not detected in the samples. It is assumed that high concentration of the malonyl-, acetyl-, and glycoside genistin in the unfermented soymilk led to the high

concentration of transformed isoflavone aglycone genistein. The mass balance study of isoflavones during the 8 weeks of storage at different temperatures showed that there was no significant reduction ($P < 0.01$) in the total isoflavone concentrations at all the storage temperatures (Tables 6.1).

There was a significant loss ($P < 0.01$) in the concentration of genistin in soymilk fermented with *L. acidophilus* ATCC 4962 during storage with the concentration of the glycoside falling from 1.56 ng/ μ L to 0.78 ng/ μ L. There was also a reduction of 36.2% ($P < 0.01$) in the level of genistin in soymilk fermented with *L. acidophilus* 4461 at during the same storage period. In contrast, there was no reduction in glycoside genistin in fermented soymilk containing *L. acidophilus* ATCC 33200 during storage. There was a significant ($P < 0.01$) reduction in the concentration of daidzin in soymilk fermented with *L. acidophilus* ATCC 4962, while the compound remained stable in fermented soymilk containing *L. acidophilus* 4461 and *L. acidophilus* ATCC 33200. Interestingly, glycitin showed much better stability as compared to daidzin and genistin in soymilk fermented with each micro-organism. The reduction in the concentration of isoflavone glycosides could have been primarily influenced by the stability of β -glucosidase from the micro-organism. The extent of reduction in the concentration of isoflavone glycosides during storage could therefore be the cumulative result of the hydrolytic action during incubation as well as the minimal biotransformation occurring during storage. The small differences in the concentration of the isoflavone glycosides in the presence of each of the 3 micro-organisms could be due to the relative hydrolytic indices of the individual micro-organisms (Chapter 4.0).

As shown in Table 6.1, there was a significant decrease ($P < 0.01$) in the concentration of genistein during storage in fermented soymilk with *L. acidophilus* ATCC 4962 and *L. acidophilus* ATCC 33200 while a non-significant reduction ($P < 0.01$) in the concentration of genistein in fermented soymilk with *L. acidophilus* 4461 occurred. On the other hand, there was a significant reduction ($P < 0.01$) in the concentration of glycitein, but only with fermented soymilk containing *L.*

acidophilus 4461. A decrease in the concentration of genistein of 19.1% and 14.2% occurred in the presence of *L. acidophilus* ATCC 33200 and *L. acidophilus* 4962, respectively. Daidzein, exhibited minimal losses over the same storage period of 18.1%, 16.2% and 5.1% in soymilk fermented with *L. acidophilus* 4461, *L. acidophilus* ATCC 4962 and of *L. acidophilus* ATCC 33200, respectively during storage at -80°C. Overall, daidzein appeared to be more stable than genistein and glycitein. The cumulative effects of individual isoflavone degradations during storage influenced the variations in concentration of the total isoflavones in the soymilk at the end of the storage period. Comparatively, *L. acidophilus* 4461, *L. acidophilus* ATCC 4962, and *L. acidophilus* ATCC 33200 led to a reduction in total isoflavones of 19.6%, 18.1% and 19.9%, respectively.

The isoflavone glycoside glycitin appeared to be more stable compared to daidzin and genistin in the presence of each *L. acidophilus* strain during storage at 4°C as was the case during storage at -80°C. Daidzein appeared to be more stable than genistein and glycitein during storage in soymilk at 4°C in the presence of each *L. acidophilus* strain, similar to the trend of results obtained during storage at -80°C. The other similarity between the two low storage temperature (-80°C and 4°C) was that genistein was very unstable in soymilk in the presence of *L. acidophilus* ATCC 33200 strain, while this compound remained fairly stable in the presence of *L. acidophilus* 4461 and *L. acidophilus* ATCC 4962. Equol may never be detected in an invitro system, but is detectable from physiological fluids such as blood plasma and urine using most analytical techniques including HPLC (Jenkins *et al.*2002). The percentage reduction in concentration of total isoflavones during storage at 4°C was 15.6%, 16.3% and 20.7% in the presence of *L. acidophilus* 4461, *L. acidophilus* 4962 and *L. acidophilus* 33200, respectively.

The changes in isoflavone concentration in fermented soymilk with the 3 *L. acidophilus* strains during storage at higher temperatures (25°C and 37°C) are also shown in Table 6.1. Glycoside glycitin remained stable in the fermented soymilk in the presence of each micro-organism during

storage at 25°C similar to that at -80°C and 4°C. Glycosides daidzin and genistin did not appear to be as stable as glycitin during storage at 25°C; however, there was a significant reduction ($P<0.01$) in the concentration of daidzin and genistin in soymilk in the presence of *L. acidophilus* ATCC 4962. Even-though there was a reduction in the concentration of isoflavone aglycones during storage at 25°C, they appeared to be in general more stable than the isoflavone glycosides. Of the three aglycones, there was a significant reduction ($P<0.01$) in the concentration of genistein only in the presence of *L. acidophilus* 4461 and *L. acidophilus* ATCC 33200. Comparatively, the percentage losses in the concentration of total isoflavones during storage at 25°C were 15.0%, 14.7% and 13.5% compared to those at 37°C which were 18.3%, 19.0% and 18.6% with *L. acidophilus* 4461, *L. acidophilus* ATCC 4962 and *L. acidophilus* ATCC 33200, respectively. During storage at 37°C, there was a significant reduction ($P<0.01$) in the concentration of genistein in soymilk in the presence of each micro-organism. In contrast, daidzein appeared to be fairly stable in fermented soymilk at this storage temperature with each *L. acidophilus* strain. There was also a significant reduction ($P<0.01$) in the concentration of aglycone glycitein only in the presence of *L. acidophilus* ATCC 4962. Isoflavone glycosides genistin, glycitin and daidzin showed significant losses ($P<0.01$) in the soymilk in the presence of *L. acidophilus* 4461 during storage at 37°C. The same occurred for only genistin in the presence of *L. acidophilus* ATCC 4962.

6.3.2 Degradation rates of isoflavone glycosides and aglycones as influenced by storage temperature

The kinetics of the individual isoflavone compounds were determined by analysing their concentrations in soymilk samples stored at various temperatures (-80°C, 4°C, 25°C and at 37°C). The reduction in the concentration of isoflavone glycoside genistin has been determined

recently in soymilk and it was demonstrated that its decrease appeared to be of first order of kinetics (Eissen *et al.*2003). Based on these previous studies, first order kinetic model was used to study the degradation constants of isoflavones in fermented soymilk with *Lactobacillus* strains during storage at the four different storage temperatures. The degradation constant was the loss of the isoflavone per day in the product at a specific storage temperature. Figures 6.1, 6.2, 6.3 and 6.4 show some first order of kinetics models of isoflavones in fermented soymilk during storage at -80°C, 4°C, 25°C and 37°C, respectively with the specified *Lactobacillus* strain. In all the storage temperatures, isoflavone aglycones, daidzein, genistein and glycitein showed less degradation than isoflavone glycosides, daidzin, genistin and glycitin. In addition, we also noted that aglycone daidzein had the least degradation followed by genistein and then glycitein in this consistent order at all storage temperatures and with each *L. acidophilus* strain. As for isoflavone glycosides, daidzin showed highest degradation in soymilk during storage at lower temperatures of -80°C (Figure 6.1) and 4°C (Figure 6.2) with each *L. acidophilus* strain. In contrast, genistin showed highest degradation during storage at higher temperatures (25°C and 37°C) with each *L. acidophilus* strain (Figures 6.3 and 6.4). The total isoflavone content showed low degradation at all storage temperatures (Figures 6.1 to 6.4), only more compared to those of individual isoflavone aglycones daidzein and genistein. On this basis, our findings confirmed that total isoflavones are generally stable in fermented soymilk during storage but that each isoflavone compound degrades differently depending on the storage temperature and the *L. acidophilus* strain.

6.3.3 Degradation rates of isoflavone compounds in soymilk as influenced by *L. acidophilus* strain and molecular structure

The first order degradation constants for the individual isoflavone glycosides and aglycones at all the storage temperatures for up to 8 weeks (56 d) with each *L. acidophilus* strain is shown in Table 6.2. The isoflavone aglycones of glycitein, daidzein and genistein showed smaller degradation constants in fermented soymilk at lower storage temperatures (-80°C and 4°C) and higher degradation constants at higher storage temperatures (25°C and 37°C) with each strain. In contrast, isoflavone Glycosides glycitin and daidzin showed relative higher degradation at lower storage temperatures (-80°C and 4°C) and lower degradation at higher storage temperatures (25°C and 37°C) in the presence of each *L. acidophilus* strain. Glycoside genistin on the other hand, surprisingly showed lower degradation at lower storage temperatures (-80°C and 4°C) and higher degradation at higher storage temperatures (25°C and 37°C) in the presence of each *L. acidophilus* strain. The unique degradation pattern with regard to genistin in fermented soymilk, which was in contrast to the degradation pattern of glycitin and daidzin, remains unclear at this present time, but could be due to a combination of various underlying factors. A possible explanation could be that at higher storage temperatures (25°C and 37°C), there is higher β -glucosidase activity than at lower storage temperatures (-80°C and 4°C) leading to higher rate of genistin de-conjugation, hence a higher rate of degradation, while at lower storage temperatures (-80°C and 4°C) there is reduced hydrolytic action of the enzyme, hence less degradation of the isoflavone glycoside. The other possible reason could be that in the fermented soymilk from SPI 590, the concentration of genistin is very high (53.1%) compared to that of glycitin (5.2%) and daidzin (20.5%) (Chapter 4.0). Therefore any slight change affecting the hydrolytic action of β -glucosidase would have a wider impact in the degree of de-conjugation of genistin as compared to daidzin and glycitin. The third possible explanation could be a preferential hydrolysis of

genistin by the endogenous β -glucosidase under optimum temperature conditions. Similar observations have recently been made by Ismail & Hayes (2005) who noted that *E. coli* β -glucosidase and almond β -glucosidase both apparently had higher affinity for genistin and daidzin as compared to glycitin, leading to significant increase in the percent hydrolysis of both daidzin and genistin than glycitin. Eissen and others (Eissen *et al.* 2003) had observed a higher degradation constant for genistin in soymilk during a 6-month storage experiments at ambient conditions using first order of kinetics.

It was also observed (Table 6.2) that aglycone daidzein had the least degradation constants while glycitein consistently had the highest degradation among the three isoflavone aglycons. This shows that daidzein is more stable than genistein while glycitein is the least stable in fermented soymilk under the storage conditions in this study. The stability of these compounds could be influenced by their molecular structural arrangements. Daidzein also known as 4', 7-dihydroxyisoflavone has two hydroxyl groups at position 4' and 7', while genistein (4', 5, 7-trihydroxyisoflavone) has 3 hydroxyl groups at positions 4', 5' and 7' of their rings. It is suggested that the increased number of hydroxyl groups in the aromatic ring A of genistein has a destabilising effect. Glycitein, however, has two hydroxyl groups at position 4' and 7' and in addition a methoxy group at position 6 of its A ring, hence is also referred to as 4',7-dihydroxy-6-methoxyisoflavone. It appears that every functional group attached to the isoflavone ring in a given position determined the relative stability of the particular isoflavone compound. This is because every group has different bond dissociation energies, which is the amount of energy required to homolytically fracture the chemical bond and are measured in kilojoules per mole or kilocalories per mole (Wingrove & Carat, 1981). It is possible that genistein is generally less stable than daidzein in fermented soymilk due to the higher number of hydroxyl groups and extra positioning and close proximity of the hydroxyl groups at positions 4' and 5'. However, a methoxy group, which is at position 6' of the aromatic ring in glycitein, could have less bond

dissociation energy compared to a hydroxyl group thereby making the isoflavone aglycone much less stable compared to daidzein and genistein in fermented soymilk. Various stability studies on polyphenols have shown that increased stability is associated with a reduced number of hydroxyl groups (Komatsu *et al.* 1993). Epigallocatechin gallate (EGCG) and epigallocatechin (EGC) were found to be less stable than epicatechin (EC) and epicatechin gallate (ECG). EGCG and ECG have a similar structural backbone except for an additional hydroxyl group at position 5' of the former. By a similar observation, the structures of EGC and EC are the same except for an additional hydroxyl group at position 5' in EGC. Perhaps, the three adjacent hydroxyl groups at position 3', 4' and 5' in EGCG and EGC were more vulnerable to destruction than the two adjacent hydroxyl groups at position 3' and 4' in ECG and EC (Zhu *et al.* 1997).

6.3.4 Mechanisms of isoflavone degradation in fermented soymilk at different storage temperatures

The overall effect of storage temperature on the mean degradation rates of isoflavones in fermented soymilk using the 3 *L. acidophilus* strains is shown in Figure 6.5. The isoflavone glycoside genistin exhibited the highest degradation in fermented soymilk during storage at 37°C, while daidzein showed the least degradation during storage in fermented soymilk at -80°C. The storage temperature at 37°C, being optimum for β -glucosidase activity resulted into maximum biotransformation of isoflavone glycosides, leading to a higher rate of degradation of these compounds. On the other hand, during storage at -80°C, due to lower activity of β -glucosidase a comparatively lower isoflavone aglycone transformation occurred than at higher storage temperatures. In general, enzymes function very slowly at subfreezing temperatures and their activities increase as the temperature is increased (Richardson & Hyslop, 1985). Most enzymes show 'optimal activity' in the 30-40°C range and begin to denature above 45°C. Thus it was

observed that the lower the storage temperature, the less degradation of isoflavone aglycones occurred and the higher the storage temperature, the more degradation of isoflavone aglycones took place. There was a consistent decrease in the rate of degradation of daidzin and glycitin with an increase in the storage temperature. It is important to note that degradation of both isoflavone glycosides and aglycones in fermented soymilk during storage was entirely dependent on the stability of the endogenous β -glucosidase from the 3 *L. acidophilus* strains. Low storage temperatures enabled better stability of the enzyme, leading to a sustained hydrolysis of isoflavone glycosides over the storage period. This possibly resulted in a higher degradation of isoflavone glycosides at lower storage temperature. At higher storage temperatures, there appeared to be a higher β -glucosidase activity, however, the activity of the enzyme was not stable over the storage period due to high cellular activity and metabolism (Bruno & Shah, 2003; Chapter 3.0). The lack of stability of the enzyme at the higher storage temperatures possibly resulted in a decrease in the hydrolytic potential, therefore a reduced rate of degradation of isoflavone glycosides in fermented soymilk. Isoflavone degradation in soymilk is primarily influenced by the storage temperature and the molecular configuration of the compound. However, the reason for the apparent greater degradation of isoflavone glycosides as compared to that of aglycones is the influence of the hydrolysing β -glucosidase enzyme. Stability studies of these compounds during storage are important for the design of storage conditions of the product to guarantee minimal losses of the bioactive components, which could consequently confer health benefits to the consumer. However, a linear model such as first order kinetics appear to offer the best option for determining the rate of degradation of isoflavone compounds in fermented soymilk and can therefore be used as a guide in determining best storage conditions for a product containing bioactive isoflavone aglycones.

6.4 CONCLUSIONS

Both the isoflavone glycosides and aglycones were found to be stable in soymilk fermented using each *L. acidophilus* strain at the storage temperatures used. Using the first order of kinetics model to determine the degradation constants at such storage temperatures, a much better understanding of the individual isoflavone degradation in the presence of probiotic *L. acidophilus* was obtained. Bioactive aglycones had much smaller degradation constants as compared to the glycoside isoflavone components. Of the isoflavone aglycones, daidzein was found to be the most stable followed by genistein, while glycitein was least stable. The stability of the isoflavone aglycones could be due to their structural composition and molecular arrangements. *L. acidophilus* strains showed preferential hydrolytic affinity to certain isoflavone glycosides, especially genistin in fermented soymilk. This ability could be important in targeting specific bioactive isoflavone aglycones and their metabolites. Storage temperature played a major role in regulating the rate of degradation of enzyme – induced soy isoflavones in fermented soymilk. Therefore, for better stability of the bioactive isoflavone aglycones of daidzein, glycitein and genistein in fermented soymilk, lower storage temperatures, particularly 4°C are most suitable and practical.

Table 6.1 Concentration of isoflavones (ng/ μ L) in fermented soymilk using 3 *L. acidophilus* strains during storage at different temperatures

Isoflavone compounds	Micro-organisms	Initial concentration ¹	Final Isoflavone Concentration at Week 8			
			-80°C	4°C	25°C	37°C
Glycitin	<i>LA</i> 4461	1.28 ^a ± 0.02	0.61 ^a ± 0.01	0.51 ^a ± 0.01	0.60 ^a ± 0.02	0.57 ^a ± 0.02
	<i>LA</i> 4962	1.12 ^a ± 0.01	0.64 ^a ± 0.01	0.49 ^a ± 0.01	0.78 ^a ± 0.03	0.95 ^a ± 0.01
	<i>LA</i> 33200	1.54 ^a ± 0.02	0.29 ^a ± 0.07	1.11 ^a ± 0.04	0.67 ^a ± 0.03	1.09 ^a ± 0.02
Daidzin	<i>LA</i> 4461	1.23 ^a ± 0.04	0.64 ^a ± 0.01	0.37 ^b ± 0.01	0.52 ^b ± 0.01	0.69 ^{ab} ± 0.01
	<i>LA</i> 4962	1.17 ^a ± 0.02	0.53 ^a ± 0.01	0.42 ^a ± 0.01	0.45 ^b ± 0.01	0.66 ^a ± 0.03
	<i>LA</i> 33200	1.36 ^a ± 0.02	0.94 ^a ± 0.04	0.59 ^a ± 0.01	0.85 ^a ± 0.01	0.73 ^a ± 0.01
Genistin	<i>LA</i> 4461	1.27 ^a ± 0.04	0.81 ^a ± 0.02	0.57 ^a ± 0.01	0.53 ^a ± 0.01	0.57 ^a ± 0.01
	<i>LA</i> 4962	1.56 ^a ± 0.02	0.78 ^b ± 0.02	0.52 ^b ± 0.02	0.41 ^b ± 0.02	0.53 ^b ± 0.01
	<i>LA</i> 33200	2.15 ^a ± 0.02	1.52 ^a ± 0.01	1.27 ^a ± 0.01	0.74 ^a ± 0.04	0.46 ^a ± 0.02
Glycitein	<i>LA</i> 4461	2.15 ^a ± 0.01	1.39 ^a ± 0.01	1.13 ^{ab} ± 0.01	1.91 ^a ± 0.01	1.63 ^a ± 0.02
	<i>LA</i> 4962	2.22 ^a ± 0.02	1.21 ^a ± 0.02	1.28 ^a ± 0.07	1.41 ^a ± 0.03	1.09 ^{ab} ± 0.01
	<i>LA</i> 33200	1.75 ^a ± 0.02	1.30 ^a ± 0.03	1.38 ^a ± 0.02	1.13 ^a ± 0.01	1.01 ^a ± 0.01
Daidzein	<i>LA</i> 4461	10.69 ^a ± 0.01	8.75 ^a ± 0.07	9.50 ^a ± 0.05	8.52 ^a ± 0.04	10.35 ^a ± 0.07
	<i>LA</i> 4962	10.09 ^a ± 0.09	8.45 ^a ± 0.03	8.93 ^a ± 0.08	8.47 ^a ± 0.08	8.25 ^a ± 0.05
	<i>LA</i> 33200	9.03 ^a ± 0.02	8.57 ^a ± 0.02	8.62 ^a ± 0.02	8.79 ^a ± 0.02	8.77 ^a ± 0.03
Genistein	<i>LA</i> 4461	30.52 ^a ± 0.01	25.35 ^a ± 0.09	27.70 ^a ± 0.06	27.97 ^a ± 0.08	24.68 ^a ± 0.09
	<i>LA</i> 4962	30.51 ^a ± 0.04	26.19 ^{ab} ± 0.07	27.46 ^a ± 0.06	28.28 ^a ± 0.07	26.32 ^a ± 0.07
	<i>LA</i> 33200	30.63 ^a ± 0.02	24.78 ^{af} ± 0.07	23.96 ^b ± 0.13	27.98 ^b ± 0.04	25.75 ^b ± 0.14
Equol	<i>LA</i> 4461	ND ²	ND ²	ND ²	0.04 ^a ± 0.00	0.01 ^a ± 0.00
	<i>LA</i> 4962	ND ²	ND ²	ND ²	ND ²	0.01 ^a ± 0.00
	<i>LA</i> 33200	ND ²	0.08 ^a ± 0.001	ND ²	0.01 ^a ± 0.00	0.01 ^a ± 0.00
Sum total	<i>LA</i> 4461	47.14 ^a ± 0.01	38.05 ^b ± 0.08	39.79 ^b ± 0.08	40.07 ^{ab} ± 0.18	38.51 ^b ± 0.02
	<i>LA</i> 4962	46.67 ^a ± 0.15	37.80 ^b ± 0.01	39.05 ^b ± 0.09	39.81 ^{ab} ± 0.17	37.81 ^b ± 0.03
	<i>LA</i> 33200	46.46 ^a ± 0.02	38.48 ^{ab} ± 0.01	36.83 ^b ± 0.04	40.17 ^b ± 0.14	37.81 ^b ± 0.09

Results expressed as mean ± standard error in ng/ μ L of fermented soymilk (n = 6). Statistical analysis by means of one-way ANOVA.

^{a,b,c,d,e,f}Means in the same row with different lowercase superscripts are significantly different (P < 0.01).

¹Initial concentration – is the concentration of isoflavones at peak β -glucosidase activity corresponding with maximum biotransformation of the isoflavones.

²ND - Not detected. *Significant (P<0.01). *LA* – *Lactobacillus acidophilus*. Sum total – Sum of all glycitin, daidzin, genistin, glycitein, daidzein, genistein and equol.

Table 6.2 First order kinetic parameters for degradation of soy isoflavones in fermented soymilk using 3 strains of *Lactobacillus acidophilus* during storage at -80°C, 4°C, 25°C and 37°C

Isoflavone compounds	Storage temperature (°C)	Rate constants ($\kappa \times 10^2 \text{ day}^{-1}$)		
		<i>L. acidophilus</i> 4461	<i>L. acidophilus</i> ATCC 4962	<i>L. acidophilus</i> ATCC 33200
Glycitin	-80	1.94	1.74	1.24
	4	1.59	1.69	1.18
	25	1.21	0.99	1.17
	37	1.11	0.31	0.99
Daidzin	-80	2.64	2.23	1.89
	4	1.72	2.16	1.62
	25	1.69	2.09	1.29
	37	1.67	1.34	1.23
Genistin	-80	1.31	1.82	1.02
	4	1.83	2.20	1.69
	25	1.88	2.64	2.33
	37	2.08	3.10	3.48
Glycitein	-80	0.88	1.25	0.64
	4	0.98	1.43	0.72
	25	1.04	1.62	0.87
	37	1.81	2.09	0.95
Daidzein	-80	0.18	0.08	0.03
	4	0.24	0.14	0.04
	25	0.25	0.19	0.08
	37	0.35	0.23	0.10
Genistein	-80	0.30	0.35	0.36
	4	0.32	0.34	0.43
	25	0.34	0.35	0.44
	37	0.44	0.36	0.50
Total isoflavones	-80	0.41	0.47	0.47
	4	0.45	0.43	0.44
	25	0.43	0.46	0.43
	37	0.48	0.46	0.49

Rate constant (κ) – is the amount of isoflavone losses in ng/ μ L of soymilk per day (day^{-1}) at a specific storage temperature

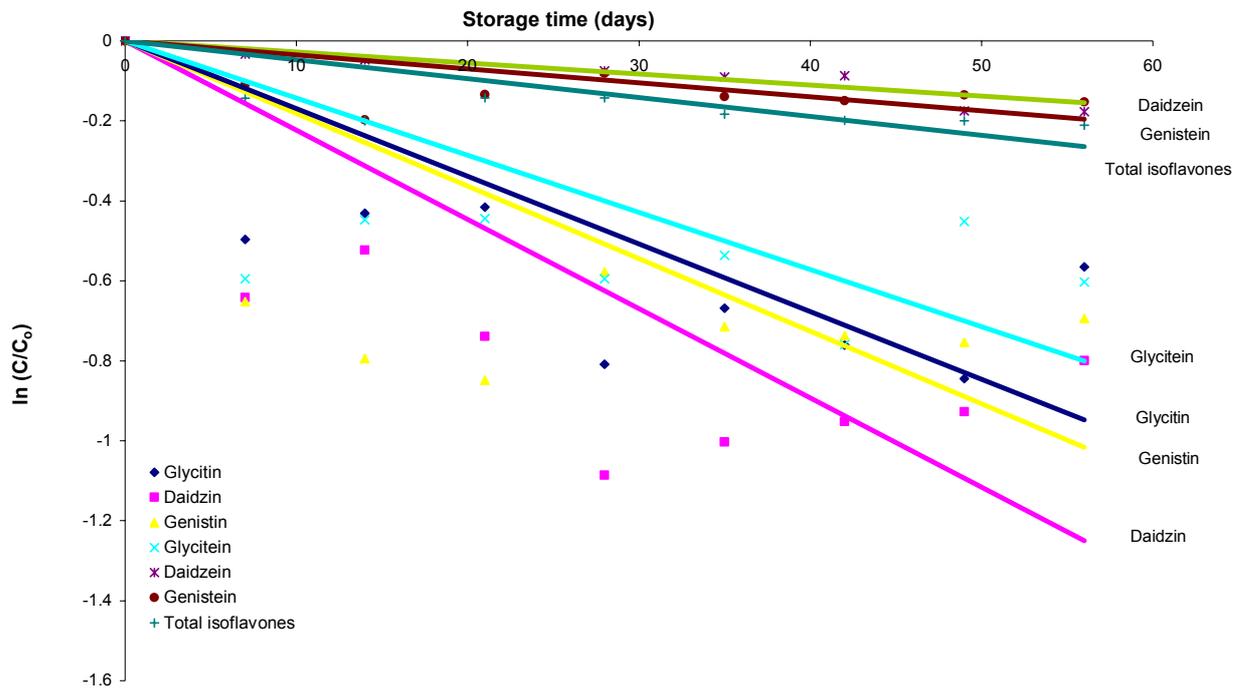


Figure 6.1 Isoflavone degradation in fermented soymilk using *L. acidophilus* ATCC 4962 during storage at -80°C . $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone isomer to its initial concentration at the specified storage temperature

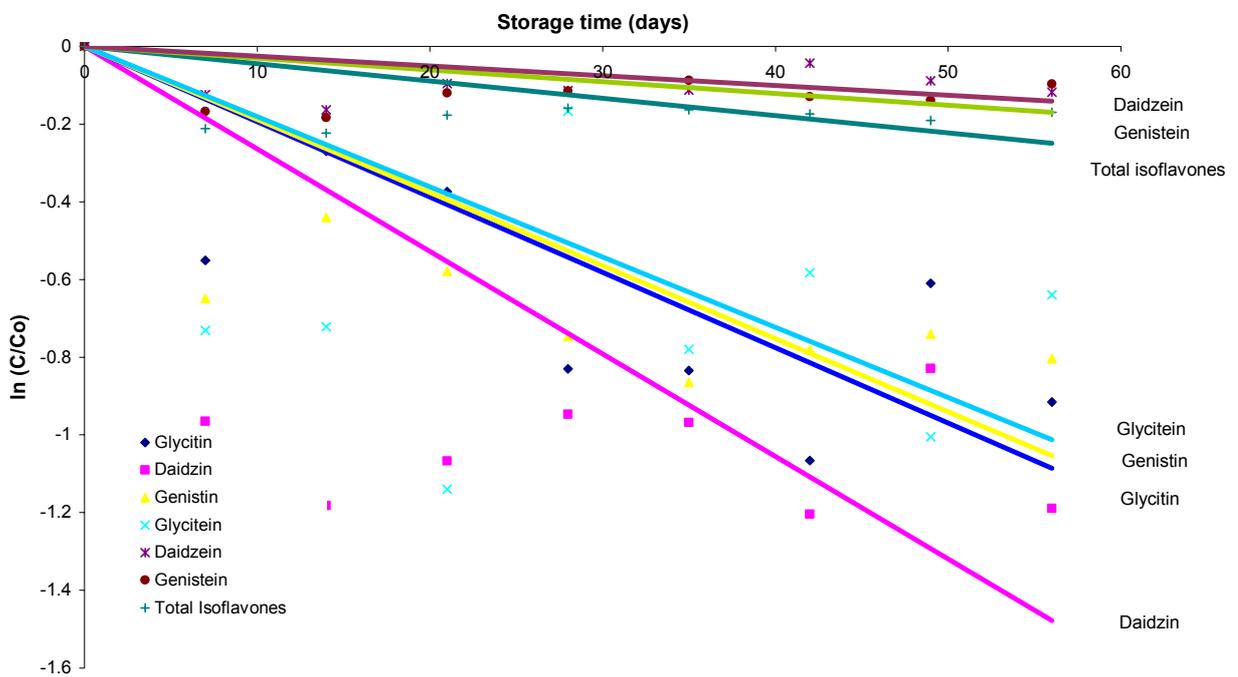


Figure 6.2 Isoflavone degradation in fermented soymilk using *L. acidophilus* 4461 during storage at 4°C. $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone isomer to its initial concentration at the specified storage temperature

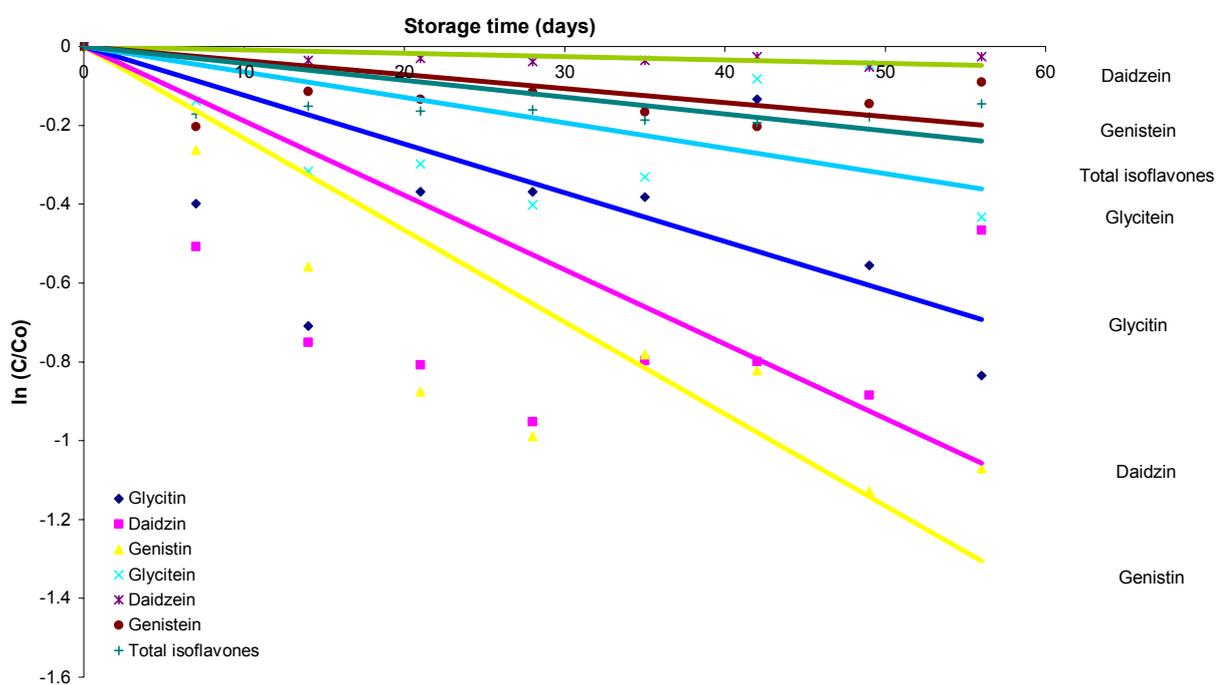


Figure 6.3 Isoflavone degradation in fermented soymilk using *L. acidophilus* ATCC 33200 during storage at 25°C. $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone isomer to its initial concentration at the specified storage temperature

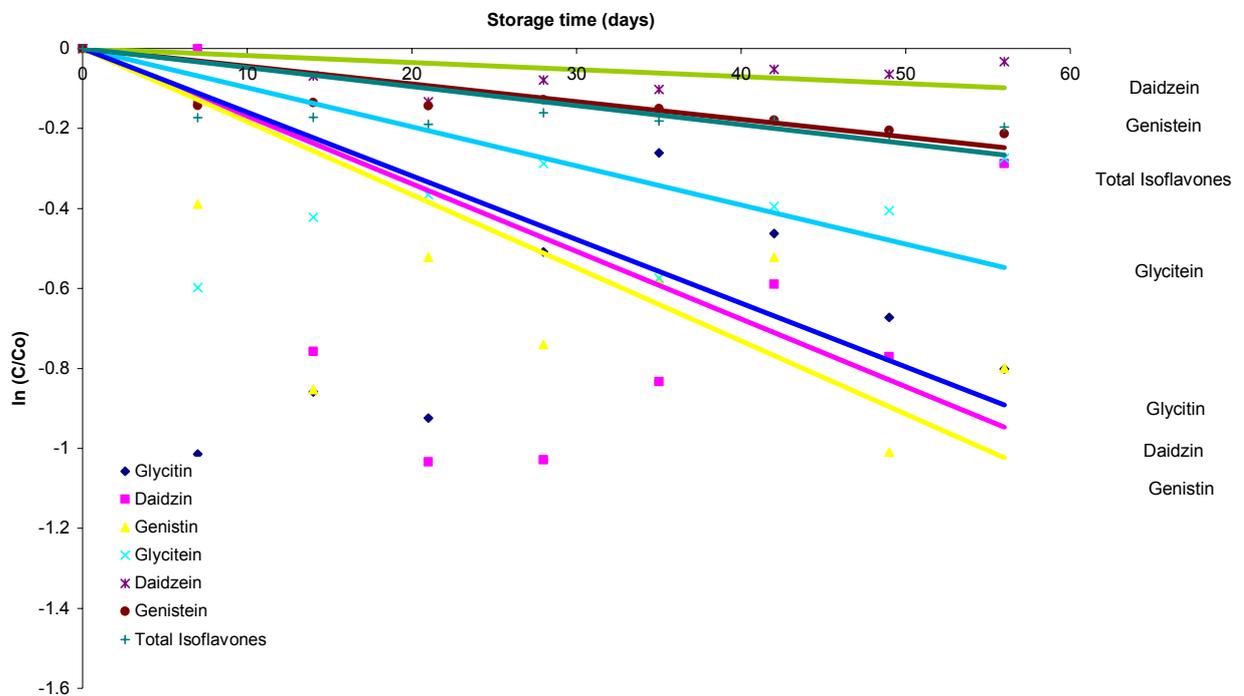


Figure 6.4 Isoflavone degradation in fermented soymilk using *L. acidophilus* ATCC 4461 during storage at 37°C. $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone isomer to its initial concentration at the specified storage temperature

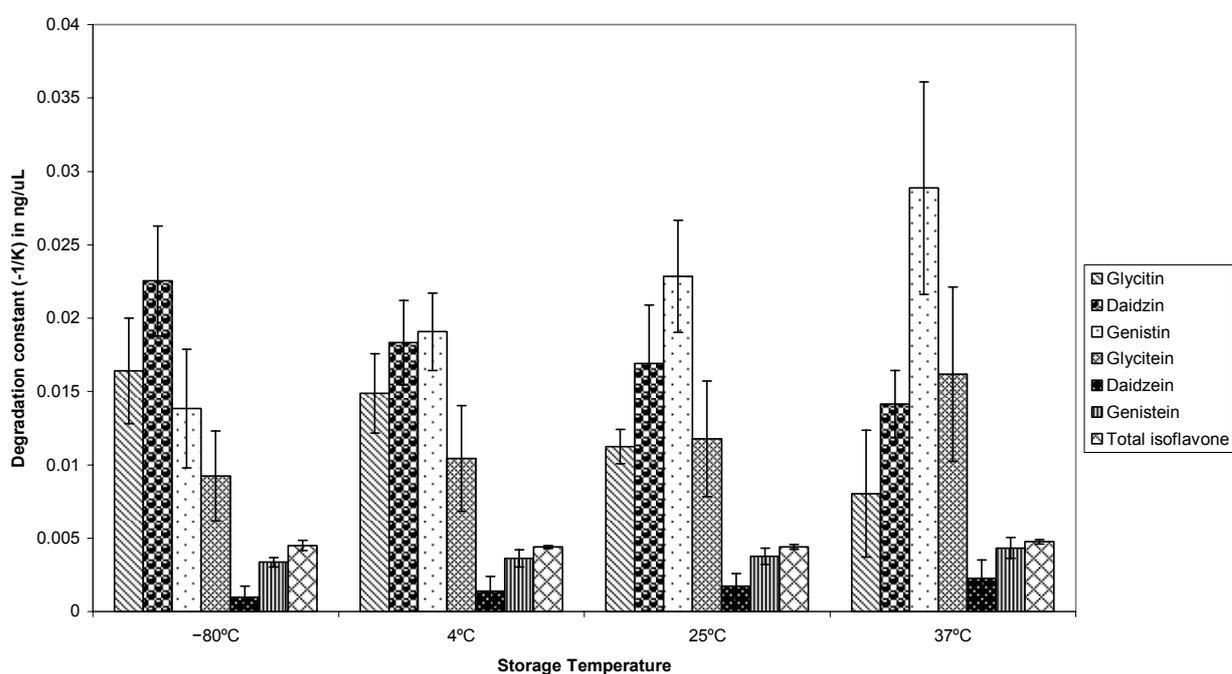


Figure 6.5 Effect of storage temperature on the degradation of isoflavones in fermented soymilk. Degradation constant ($-1/\kappa$) is the rate of degradation of the isoflavone compound in fermented soymilk at the specified storage temperature

7.0 Role of microbial strain and storage temperatures in the degradation of isoflavone phytoestrogens in fermented soymilk with selected β -glucosidase producing *Lactobacillus casei* strains

7.1 INTRODUCTION

The predominant, fraction of the isoflavones found in soybeans and soybean extracts, especially in unprocessed or mildly processed soybean products, occurs not as the glycoside forms but rather as their 6"-O-malonyl esters. Chemically, these compounds consist of the glycoside form esterified with a single malonate half-ester, located at the primary (6"-) hydroxy group of the sugar moiety. This remarkable development has introduced some new uncertainties as to the pharmacologically active components of soy products. Moreover, further complicating matters, the malonyl esters are thermally and chemically unstable and are easily converted during soy product processing, especially via heating, toasting and/or high pH, to either the free glycoside form or to yet another type of isoflavone derivative, namely the 6"-O-acetyl esters, which are somewhat more stable than the malonyl forms.

There are approximately 230 individual types of isoflavones and most are commonly found in legumes, with the highest amount found in soybeans (Cassidy *et al.* 2000). The parent isoflavones are the aglycone structures of daidzein, genistein and glycitein, which are conjugated to form malonyl-, acetyl- and β -glycoside configurations. The structural similarity of aglycons to the mammalian oestrogen oestradiol-17 β enables them to mimic the function of oestradiol in the human body (Setchell & Cassidy, 1999). Due to this similarity, they share several features in common with oestradiol, including a pair of hydroxyl groups separated by a similar distance (Miksicek, 1995) and the presence of a phenolic ring which is a prerequisite for binding to the oestrogen receptor (Metzger *et al.* 1995). The chemical form of the isoflavone and its metabolites

influence the extent of absorption; aglycone form is more readily absorbed and bioavailable than the highly polar conjugated species (Setchell, 2000; Izumi *et al.* 2000). In contrast, most recent studies reported that the bioavailability of daidzein and genistein isoflavones was greater when ingested as glycosides rather than as aglycones (Setchell *et al.* 2001). Two other studies found no significant differences in the absorption efficiency of aglycones and glycosides (Richelle *et al.* 2002; Zubik & Meydani 2003).

There is considerable evidence supporting a decreased risk of developing breast cancer in countries with increased consumption of soy-based foods (Cassidy, 1996; Wiseman, 1997). Cross cultural studies have demonstrated lower mortality from breast cancer in Japan than in the United States (Kelsey & Horn-Ross, 1993). *L. casei*, *L. acidophilus* and *Bifidobacterium* spp. are important members of the intestinal microflora. They possess β -glucosidase enzyme (Chapter 3.0); however, their cell viability is dependent on the storage temperature. Beneficial effects of probiotic bacteria can be expected only when viable cells are ingested (Shah, 2000). Thus the incorporation of β -glucosidase producing *L. casei* strains in soymilk may provide a combination of benefits as probiotics when viable as well as that from transformation of isoflavone glycosides to bioactive isoflavone aglycones.

Isoflavones are mostly stable and are not generally destroyed by heat but are rather subject to intra-conversion between different forms (Shimoni, 2004). Baking has been found to increase the concentration of β -glycosides while frying appears to increase the amount of acetylglycosides and aglycones (Coward *et al.* 1998). The stability of the individual isoflavone compounds including the isoflavone aglycone components has not been studied during storage at lower temperatures. It is important that once the biological activity of a soy-based product has been improved, the stability of bioactive components be maintained during the storage period in order to confer the health benefits to the consumer. Very little has so far been reported on the assessment of changes in isoflavone profile in soymilk during storage. The aims of this study

were to examine the stability of isoflavone glycosides and aglycones in fermented soymilk during storage at various temperatures (-80°C, 4°C, 25°C and 37°C) and to establish the role of temperature and microbial strain for the degradation of isoflavone compounds in soymilk during storage.

7.2 MATERIALS AND METHODS

7.2.1 Bacteria

Pure culture of *L. casei* 2607 was obtained from the Victoria University Culture Collection (Werribee, Vic, Australia) and that of *L. casei* ASCC 290 from the Australian Starter Culture Research Centre (Werribee, Vic, Australia). The purity of the culture was confirmed through the procedure mentioned in section 3.2.1 and stored in circumstances similar to those in section 3.2.1 as well.

7.2.2 Soymilk manufacture

Soy protein isolate (SPI; SUPRO 590), supplied by The Solae Co. (Chatswood, NSW, Australia), was used in the production of soymilk at 4% of ultra-pure distilled water as described in section 3.2.2. After reconstitution, the soymilk was dispensed into sixteen glass bottles at 400 mL each, then autoclaved at 121°C for 15 min. The pH was adjusted in a laminar flow to 6.7 using 5 M sodium hydroxide.

7.2.3 Fermentation of soymilk with *L. casei* 2607 and *L. casei* ASCC 290

The micro-organisms were activated in rehydrated de Mann Rogosa Sharpe (MRS) broth (de Mann *et al.* 1960) at 37°C for 20 h successively 3 times followed by a fourth activation in sterile soymilk according to the method of Tsangalis *et al.* (2002). An inoculum level of 5% (v/v) and the incubation at 37°C for 20 h were used for activation. For storage studies, 16 bottles each

containing 400 mL of sterile soymilk were inoculated (in duplicate) with the active culture (5% v/v), and incubated at 37°C for 24 h to achieve peak β -glucosidase activity and to transform the predominant isoflavone glycosides to the aglycone forms as reported in our earlier study (Chapter 4.0). After fermentation, 2 bottles containing 400 mL each of the active culture were then stored at 4 different temperatures (-80°C, 4°C, 25°C, and 37°C) for 8 weeks. Fifty millilitre aliquots were withdrawn aseptically at weekly intervals and stored immediately at -80°C for analysis of isoflavones. The frozen aliquots were freeze-dried using a Dynavac® FD300 freeze drier (Rowville, Vic., Australia) for isoflavone extraction and analysis using reverse-phase high-performance liquid chromatography (HPLC).

7.2.4 Extraction of isoflavones from freeze dried samples

The extraction of isoflavones using HPLC grade methanol from freeze-dried sample and sample concentration for the HPLC runs was conducted following the procedure outlined in section 4.2.6.

7.2.5 Reversed-phase HPLC apparatus and reagents

Analyses were carried out using a Hewlett Packard® 1100 series HPLC (Agilent Technologies, Forest Hill, Vic., Australia) with autosampler, quaternary pump, diode array ultraviolet (UV) visible detector, vacuum degasser, and thermostatically controlled column compartment. Alltech Alltima (Deerfield, IL, USA) HP C18HL (250 mm \times 4.6 mm internal diameter, 5 μ m) reversed-phase C18 column attached to an Alltima HP C18HL (7.5 mm \times 4.6 mm internal diameter, 5 μ m) guard column was used to separate the isoflavone compounds was used to separate the isoflavone compounds as mentioned in section 4.3.2. HPLC-grade methanol, trifluoro-acetic acid, absolute ethanol, and ammonium acetate from Sigma and all the isoflavone standards were obtained from

sources mentioned in sections 4.2.8. All reagents used in isoflavone extraction and HPLC analyses were filtered through a 0.5 µm FH membrane (Millipore®, Bedford, MA, USA).

7.2.6 HPLC analysis of isoflavones

HPLC isocratic elution was used to isolate the isoflavones for detection and the mobile phase consisted of 100% methanol and 10 mM ammonium acetate buffer (50:50) containing 1 mL of trifluoro-acetic acid per litre of solvent mixture. This was set at a flow rate of 0.95 mL/min according to our method in Chapter 5.0 (section 5.2.8). Injection volumes of isoflavone standards and of the samples, HPLC run time, column specifications and guard column and its specifications were according to our method outlined in chapter 5.0 (section 5.2.8). A diode array UV-visible detector was set at dual wavelengths of 259 nm to detect malonyl-, acetyl-, and β-glycosides, aglycones, and flavone (ISTD), and 280 nm to detect equol as mentioned in section 5.2.8. Single standards were prepared for peak identification. Isoflavone concentrations were calculated back to wet basis (ng/µL of soymilk).

7.2.7 Isoflavone identification

The use of retention times and UV absorption patterns of pure isoflavone standards were important in the identification isoflavones in samples following extraction and HPLC analysis. Detection of all isoflavone standards as well as those in the samples occurred at the respective optimum absorption wavelengths mentioned in section 6.2.8.

7.2.8 Standard solutions and calibration curves

Crystalline pure compounds were dissolved in 100% methanol in order to make stock solutions for different isoflavone standards. Each solution was then serially diluted with methanol to 5 working solutions of different concentrations in order to prepare a standard curve. The procedure

of HPLC analysis of aliquots as well as comparison of the peak area and concentration for each standard solution was calculated and used for calibration as described in section 6.2.9. These were used to calculate the concentrations of isoflavones present in freeze dried fermented soymilk.

7.2.9 Statistical analysis of data

The quantification of isoflavones and pH measurements were performed in duplicate on 3 trials and repeated. The data are means of 6 measurements and are presented as a mean \pm standard error of 6 analyses. To find the difference in isoflavone concentrations in soymilk at different temperatures, means of isoflavone concentrations at successive weeks of storage were analysed using one-way analysis of variance (ANOVA) and 99% confidence levels using Microsoft[®] Excel Statpro[®] as described by Albright *et al.* (1999). ANOVA data with a $P < 0.01$ was classified as statistically significant.

7.3 RESULTS AND DISCUSSION

7.3.1 Isoflavone concentrations at peak β -glucosidase activity

The isoflavone concentrations determined at peak β -glucosidase activity during incubation at 37°C were regarded as the initial concentrations as shown in Tables 7.1. Indogenous β -glucosidase from the *L. casei* strains was responsible for the biotransformation of isoflavones in soymilk by hydrolysing the 1, 6 β -glycoside bonds of the predominant isoflavone glycosides in unfermented soymilk (Chapter 3.0; Chapter 4.0). The extent of hydrolysis was dependent on the hydrolytic potential of the endogenous β -glucosidase from the micro-organism (Chapter 4.0). There was some variation in the initial concentration of individual isoflavones in soymilk fermented with each of the *L. casei* strains. Genistein occurred in the highest concentrations followed by daidzein and then glycitein, while there was a decrease in isoflavone glycosides such

as glycitin, daidzin and genistin. It is assumed that high concentration of the malonyl-, acetyl-, and glycoside genistin in the unfermented soy milk led to the high concentration of aglycone genistein through biotransformation.

7.3.2 Concentration of isoflavones in fermented soymilk during storage

While the concentration of most of the individual isoflavones decreased, the reduction was not significant ($P < 0.01$), indicating a general stability of the isoflavones under the storage conditions. The concentration of the individual isoflavones as well as that of total isoflavones in fermented soymilk during storage at -80°C is shown in Table 7.1. There was a significant reduction ($P < 0.01$) in the concentration of glycitin and genistin in soymilk fermented with both *L. casei* 2607 and *L. casei* ASCC 290. Daidzin remained fairly stable in soymilk fermented with both the *L. casei* strains; however, the decrease in concentration was not significant ($P < 0.01$). The reduction in the concentration of isoflavone glycosides could have been primarily influenced by the action of β -glucosidase from the micro-organisms. During storage at -80°C , it was expected that the enzyme activity would be minimal, thus slowing down the hydrolytic action of endogenous β -glucosidase, therefore reducing the biotransformation of the residual isoflavone glycosides. Of the three main isoflavone aglycones, daidzein appeared to be most stable, while there was no significant reduction ($P < 0.01$) in the concentration of the aglycones except for glycitein in soymilk fermented with *L. casei* ASCC 290. Daidzein showed the lowest reduction at 7.7% and 12.3% in soymilk fermented with *L. casei* ASCC 290 and *L. casei* 2607, respectively. Interestingly, there were differences in the reduction levels of isoflavone aglycones amongst the two micro-organisms, possibly due to their differences in hydrolytic potential as a result of strain specificity. It is possible that degradation of aglycones was influenced less by the microbial enzyme than the glycosides. Other factors that possibly contributed more to the aglycone degradation in the soymilk could be the storage temperature and the molecular configuration of

the isoflavone aglycones. In general, the percentage decrease in the concentration of isoflavone aglycones over the 8 weeks storage period was much less compared to that of the isoflavone glycosides. The cumulative effects of individual isoflavone degradations during storage at -80°C as influenced by the β -glucosidase from *L. casei* strains were reflected in the variations in concentration of the total isoflavones in the soymilk.

Table 7.1 also show the final concentration of isoflavones in fermented soymilk during storage at 4°C. There was a significant reduction ($P<0.01$) in the concentration of daidzin and genistin in the presence of *L. casei* 2607 while the concentration of only genistin reduced significantly in soymilk fermented with *L. casei* ASCC 290. Glycitin appeared to be more stable in soymilk fermented with the two micro-organisms, as compared to daidzin and genistin. The glycoside genistin was less stable in the presence of each of the micro-organisms during storage at 4°C and at -80°C. On the other hand, daidzin was stable in soymilk fermented with *L. casei* ASCC 290 during storage at 4°C and at -80°C. As for aglycones, glycitein and daidzein remained significantly stable ($P<0.01$) in soymilk fermented with *L. casei* ASCC 290 during storage at 4°C. On the contrary, soymilk fermented with *L. casei* 2607 during storage at the same temperature showed a significant decrease ($P<0.01$) in the concentration of glycitein and daidzein. The percentage reduction in concentration during storage at 4°C was 19.7% and 20.8% as compared to 19.6% and 18.4% during storage at -80°C in the presence of *L. casei* ASCC 290 and *L. casei* 2607, respectively. There was a greater reduction in the concentration of total isoflavones in fermented soymilk stored at 4°C than at -80°C.

The changes in isoflavone concentration in fermented soymilk with each of the *L. casei* strains during storage at room temperature (25°C) and at 37°C were also shown in Table 7.1. Glycitin remained stable in the fermented soymilk in the presence of each micro-organism during storage at 25°C, similar to that at 4°C. Genistin and daidzin were not as stable as glycitin during storage at 25°C however, there was no significant reduction ($P<0.01$) in the concentration of daidzin in

soymilk fermented with *L. casei* ASCC 290. There was a significant reduction ($P < 0.01$) in the concentration of genistein in soymilk fermented with each *L. casei* strain. However, daidzein was the most stable among the isoflavone aglycones. Glycitein showed a significant reduction ($P < 0.01$) in its concentration in soymilk fermented with *L. casei* ASCC 290 during storage at 25°C, unlike that in soymilk fermented with *L. casei* 2607 during storage at the same temperature. Total isoflavone concentration in the fermented soymilk in the presence of each *L. casei* strain was significantly ($P < 0.01$) reduced during storage at 25°C as was the case during storage at 37°C. Comparatively, the percentage losses in the concentration of total isoflavones during storage at 25°C were 19.7% and 20.8% compared to those at 37°C of 18.9% and 20.1% with *L. casei* ASCC 290 and *L. casei* 2607, respectively. During storage at 37°C, there was a significant reduction ($P < 0.01$) in the concentration of genistein in soymilk in the presence of each of the two micro-organisms. There was also a significant reduction ($P < 0.01$) in the concentration of glycitein and daidzein in soymilk fermented with *L. casei* 2607 and *L. casei* ASCC 290, respectively. The concentration of genistein reduced significantly ($P < 0.01$) in soymilk fermented with each of the *L. casei* strains during storage at 37°C. Daidzein appeared to be most stable as compared to genistein and daidzein and this trend was similar to storage at -80, 4°C and at 25°C. As for isoflavone glycosides, there was a reduction in the concentration, however, the losses were not significant ($P < 0.01$) for glycitin and daidzin but significant ($P < 0.01$) for genistin in soymilk fermented with each *L. casei* strain during storage at 37°C. Part of the reason for lesser stability of isoflavone glycosides as compared to aglycones in the soymilk could be the hydrolysing influence of microbial β -glucosidases.

7.3.3 The kinetics of soy isoflavone degradation in fermented soymilk during storage

The ability to predict the impact of storage conditions on isoflavone contents and composition is crucial for optimisation of a process. This requires obtaining reliable data on the kinetics of

isoflavone degradation under the likely storage conditions depending on the product characteristics. The kinetics of the individual isoflavone compounds were determined by monitoring their concentrations in soymilk stored at various temperatures (-80°C, 4°C, 25°C and at 37°C) over the storage period. The reduction in the concentration of isoflavone glycoside genistin was recently determined in soymilk and it was demonstrated that its decrease was of first order of kinetics (Eissen *et al.* 2003). Based on these previous studies, first order kinetic model was used to study the degradation of individual isoflavone glycosides and aglycones at all the storage temperatures for up to 8 weeks (56 d) with each *L. casei* strain. Using the concentrations of glycosides and aglycones during peak β -glucosidase activity as initial concentrations (C_0), kinetic degradation constants was calculated based on the weekly concentrations of the isoflavones in the soymilk. Some first order of kinetics models of isoflavones in fermented soymilk during storage at -80°C, 4°C, 25°C and 37°C, are shown in Figures 7.1, 7.2, 7.3 and 7.4 respectively, with the specified *Lactobacillus casei* strain. In all the storage temperatures, isoflavone aglycones, daidzein, genistein and glycitein showed less degradation than isoflavone glycosides, daizin, genistin and glycitin. In addition, we also noted that aglycone daidzein had the least degradation followed by genistein and then glycitein in this consistent order at all storage temperatures and with each *L. casei* strain. As for isoflavone glycosides, daidzin showed highest degradation in soymilk during storage at lower temperatures of -80°C (Figure 7.1) and 4°C (Figure 7.2) with each *L. casei* strain. In contrast, genistin showed highest degradation during storage at higher temperatures (25°C and 37°C) with each *L. casei* strain (Figure 7.3 and 7.4). The total isoflavone content showed low degradation at all storage temperatures (Figure 7.1 to 7.4), only more compared to those of individual isoflavone aglycones daidzein and genistein. On this basis, our findings confirmed that total isoflavones are generally stable in fermented soymilk during storage but that each isoflavone compound degrades differently depending on the storage temperature and the *L. casei* strain.

7.3.4. Degradation rates of isoflavones in fermented soymilk as influenced by storage temperature and molecular structure

First order kinetic parameters for the degradation of soy isoflavones in fermented soymilk in the presence of both *L. casei* strains during storage are shown in Table 7.2. Isoflavone glycosides glycitin and daidzin showed greater degradation at lower storage temperatures (-80°C and 4°C) and lower degradation at higher storage temperatures (25°C and 37°C) in the presence of each of the microorganisms. Genistin on the other hand showed less degradation at lower storage temperatures (-80°C and 4°C) and higher degradation at higher storage temperatures (25°C and 37°C). This similar trend also occurred in soymilk fermented with each of the microorganism. The unique degradation pattern with regard to genistin, which was in contrast to the degradation pattern of glycitin and daidzin could be due to a combination of various underlying factors. A possible explanation could be that at higher storage temperatures (25°C and 37°C), β -glucosidase activity in soymilk could be higher than at lower storage temperatures (-80°C and 4°C). This leads to greater hydrolysis of glycoside genistin while at lower storage temperatures (-80°C and 4°C) the reduced hydrolytic action of the enzyme results into less degradation of the isoflavone glycoside. The other possible reason could be that in the unfermented soymilk manufacture from SPI SUPRO 590, the initial concentration of genistin is much higher (53.1%) than that of glycitin (5.2%) and daidzin (20.5%) (Chapter 4.0). Therefore any slight change affecting the hydrolytic action of β -glucosidase could have a wider impact in the degree of de-conjugation of genistin as compared to daidzin and glycitin. The third possible explanation could be a preferential hydrolysis of genistin by the endogenous β -glucosidase under optimum temperature conditions. A similar observation that *E. coli* β -glucosidase and almond β -glucosidase both apparently had higher affinity for genistin and daidzin as compared to glycitin leading to a substantial increase in the percent hydrolysis of both daidzin and genistin than glycitin has been recently reported

(Ismail & Hayes, 2005). In another study, Eissen *et al.* (2003) observed a higher degradation constant for genistin than other isoflavones in soymilk during a 6-month storage experiments at ambient conditions possibly due to the same reasons outlined. While various isoflavones showed different degradation constants at the storage temperatures, there was a little difference in the degradation constants of the sum total of isoflavones in the soymilk.

As for isoflavone aglycones glycitein, daidzein and genistein, there was smaller degradation constants obtained in fermented soymilk at lower storage temperatures (-80°C and 4°C) and bigger constants at 25°C and 37°C. This trend also occurred for each of the two microorganisms. Daidzein showed the least degradation constants while glycitein consistently had the highest degradation among the three isoflavone aglycons. We have observed a similar trend of results using *Bifidobacterium animalis* Bb12 (Chapter 5.0) and *L. acidophilus* strains (Chapter 6.0) in fermented soymilk during storage at different temperatures. The stability of these compounds could be influenced by their molecular structural configurations. Daidzein also known as 4', 7-dihydroxyisoflavone has two hydroxyl groups at positions 4 and 7, while genistein (4', 5, 7-trihydroxyisoflavone) has 3 hydroxyl groups at positions 4, 5 and 7 of their rings. Glycitein, however, has two hydroxyl groups at positions 4 and 7 and in addition a methoxy group at position 6 of its A ring, hence this compound is also referred to as 4',7-dihydroxy-6-methoxyisoflavone. Every functional group attached to the isoflavone ring in a given position is likely to determine the relative stability of the particular molecule as influenced by the group's bond dissociation energy (Wingrove & Caret, 1981). It is possible that genistein is comparatively less stable than daidzein in fermented soymilk due to the higher number of hydroxyl groups and extra positioning and close proximity of the hydroxyl groups at positions 4 and 5. Various stability studies on polyphenols have shown that increased stability is associated with a reduced number of hydroxyl groups (Komatsu *et al.* 1993). However, a methoxy group, which is at position 6 of the aromatic ring in glycitein, could have less bond dissociation energy compared to

a hydroxyl group thereby making the isoflavone aglycone much less stable compared to daidzein and genistein in fermented soymilk.

7.3.5 Overall effects of storage temperature on the stability of isoflavones in fermented soymilk

The overall mean effect of storage temperature on the stability of isoflavones in fermented soymilk using the *L. casei* ASCC 290 and *L. casei* 2607 is shown in Figure 7.5. The isoflavone glycoside genistin exhibited the highest degradation in fermented soymilk during storage at 37°C. Glycoside genistin was also the least stable of all isoflavones compounds at all the storage temperatures. Aglycone daidzein was however the most stable isoflavone at all the storage temperatures. The storage temperature at 37°C, being optimum for β -glucosidase activity resulted in maximum biotransformation of isoflavone glycosides, leading to a higher rate of degradation of these compounds. On the other hand, lower storage temperatures such as -80°C limited the activity of β -glucosidase leading to comparatively lower isoflavone aglycone transformation than at higher storage temperatures. In general, enzymes function slowly at subfreezing temperatures and their activities increase with temperature. Most enzymes show 'optimal activity' in the 30-40°C range and begin to denature above 45°C (Richardson & Hyslop, 1985). In this study, it was observed that the lower the storage temperature, the less degradation of isoflavone aglycones occurred and vice-versa. Isoflavone glycosides of daidzin and glycitin appeared to have a higher degradation at lower storage temperatures than at higher storage temperatures. There was also a consistent decrease in the rate of degradation of the two isoflavone glycosides with an increase in the storage temperature. Degradation of isoflavone glycosides in the fermented soymilk during storage appeared to be dependent on the stability of the endogenous β -glucosidase from each *L. casei* strain. Low temperatures restrict cellular activity and metabolism, therefore allowing very small energy losses, thus better stability of the cells and the enzyme within the cells (Shah, 2000;

Bruno & Shah, 2003; Chapter 3.0). The better stability of the enzyme at lower storage temperatures possibly enabled a sustained hydrolysis of isoflavone glycosides over the storage period resulting in a higher degradation of isoflavone glycosides. The lack of stability of the enzyme at the higher storage temperatures possibly resulted in a decrease in the hydrolytic potential, therefore a reduced rate of degradation of isoflavone glycosides in fermented soymilk.

7.3.6 Comparison of *L. casei* ASCC 290 and *L. casei* 2607 and their role in isoflavone degradation in soymilk during storage

Figure 7.6 shows the comparative influence of each micro-organism in the degradation of isoflavone in fermented soymilk during storage at different temperatures. Glycoside genistin showed the highest degradation in fermented soymilk with each micro-organism during storage at 4°C, 25°C and 37°C. There were, however, remarkable differences on the influence of each micro-organism in the degradation of individual isoflavone compounds. At lower storage temperatures (-80°C and 4°C), soymilk fermented with *L. casei* 2607 showed higher degradation of genistin compared to that with *L. casei* ASCC290. With increase in storage temperature, the gap between the stability of glycoside genistin in soymilk fermented with each of the two micro-organisms became narrow. The same trend occurred with the degradation of glycoside daidzin, except that storage temperature did not alter the difference between the degrees of degradation in the presence of each micro-organism. Comparative higher degradation rates of genistin and daidzin in soymilk fermented with *L. casei* 2607 than those in soymilk fermented with *L. casei* ASCC 290 suggested that the two compounds were more stable when soymilk was fermented with *L. casei* ASCC 290. It may be possible that *L. casei* 2607 had a greater hydrolytic potential than *L. casei* ASCC 290 in de-conjugating daidzin and genistin which are the predominant isoflavone glycosides in unfermented soymilk. As for glycoside glycitin, soymilk fermented with *L. casei* ASCC 290 showed higher degradation than that with *L. casei* 2607.

A direct linear relationship was observed between degradation of bioactive isoflavone aglycones and storage temperature in soymilk fermented with each micro-organism. During storage at low temperatures, lower degradation constants were obtained while at higher storage temperatures, concomitant increases in degradation constants were observed. Glycitein showed the highest degradation constant among the three main isoflavone aglycones at all storage temperatures in soymilk fermented with each strain. Comparatively, glycitein was less stable in soymilk fermented with *L. casei* 2607 compared to that of soymilk fermented with *L. casei* ASCC 290 at all storage temperatures. However, there was higher degradation rates for daidzein and genistein in soymilk fermented with *L. casei* ASCC 290 than those in soymilk fermented with *L. casei* 2607, suggesting better stability of the two aglycones in the presence of the latter. Thus it may be possible that *L. casei* 2607 could have stronger hydrolytic potential in hydrolysing daidzin and genistin glycosides than that of *L. casei* ASCC 290. *L. casei* 2607 was confirmed in our earlier studies (Chapter 3.0) as having a stronger β -glucosidase based hydrolytic potential than *L. casei* ASCC 290. On the other hand, it was observed that glycitein was less stable in soymilk fermented with *L. casei* 2607 than in soymilk fermented with *L. casei* ASCC 290 at all the storage temperatures. This unique pattern can possibly be attributed to strain specificity and its role in comparative preferential biotransformation of glycoside glycitin.

7.4 CONCLUSIONS

Both the isoflavone glycosides and aglycones were in general found to be stable in soymilk fermented with *L. casei* strains during storage. Isoflavone aglycones were more stable compared to glycosides partly due to the hydrolysing influence of the microbial enzyme on isoflavone glycosides. Of the isoflavone aglycones, daidzein was most stable followed by genistein, while glycitein was least stable. The stability of the isoflavone aglycones in the soymilk could be primarily influenced by their molecular configurations as well as the storage temperatures. *L.*

casei strains were found to have different preferential hydrolytic affinity to certain isoflavone glycosides, especially genistin in fermented soymilk. This ability could be important in targeting specific isoflavone aglycones and their metabolites. Storage temperature played a major role in regulating the rate of degradation of enzyme – induced soy isoflavones in fermented soymilk. For better stability of the isoflavone aglycones of daidzein, glycitein and genistein in fermented soymilk, lower storage temperatures are recommended. A linear model such as first order kinetics is important in determining the rate of degradation of isoflavone compounds in fermented soymilk and can therefore be used as a guide in determining best storage conditions for a product containing isoflavone glycosides and aglycones.

Table 7.1 Concentration of isoflavones (ng/μL) in fermented soymilk using two *L. casei* strains during storage at different temperatures

Isoflavone compounds	Micro-organisms	Initial concentration ¹	Final Isoflavone Concentration at Week 8			
			-80°C	4°C	25°C	37°C
Glycitin	<i>LC</i> ASCC290	1.67 ^a ± 0.02	0.70 ^a ± 0.01	1.17 ^a ± 0.01	0.81 ^a ± 0.04	0.75 ^a ± 0.02
	<i>LC</i> 2607	1.28 ^a ± 0.01	0.57 ^b ± 0.01	0.49 ^a ± 0.01	0.53 ^a ± 0.01	0.93 ^a ± 0.01
Daidzin	<i>LC</i> ASCC290	1.13 ^a ± 0.04	0.64 ^a ± 0.07	0.74 ^a ± 0.01	0.84 ^a ± 0.02	0.91 ^a ± 0.01
	<i>LC</i> 2607	1.93 ^a ± 0.02	0.71 ^a ± 0.02	0.94 ^a ± 0.01	0.73 ^a ± 0.02	0.90 ^a ± 0.01
Genistin	<i>LC</i> ASCC290	2.84 ^a ± 0.04	1.25 ^b ± 0.03	1.46 ^{ab} ± 0.03	0.98 ^a ± 0.04	1.02 ^b ± 0.03
	<i>LC</i> 2607	3.18 ^a ± 0.02	1.18 ^b ± 0.02	1.54 ^b ± 0.02	1.02 ^{bc} ± 0.01	1.09 ^b ± 0.01
Glycitein	<i>LC</i> ASCC290	2.02 ^a ± 0.01	1.50 ^a ± 0.02	1.54 ^a ± 0.02	1.36 ^a ± 0.03	1.44 ^a ± 0.01
	<i>LC</i> 2607	2.24 ^a ± 0.02	1.36 ^{ab} ± 0.02	1.28 ^a ± 0.02	1.04 ^b ± 0.01	1.35 ^{ab} ± 0.08
Daidzein	<i>LC</i> ASCC290	9.80 ^a ± 0.01	9.05 ^a ± 0.03	9.43 ^a ± 0.07	9.05 ^a ± 0.04	9.00 ^b ± 0.01
	<i>LC</i> 2607	9.53 ^a ± 0.09	8.96 ^a ± 0.03	9.17 ^a ± 0.03	9.12 ^a ± 0.02	9.08 ^a ± 0.02
Genistein	<i>LC</i> ASCC290	28.60 ^a ± 0.01	24.68 ^a ± 0.12	24.54 ^a ± 0.13	23.93 ^a ± 0.06	24.02 ^a ± 0.07
	<i>LC</i> 2607	27.85 ^a ± 0.04	25.46 ^a ± 0.05	25.49 ^a ± 0.09	25.62 ^a ± 0.02	24.29 ^{ab} ± 0.06
Equol	<i>LC</i> ASCC290	ND ²	0.13 ^a ± 0.001	0.01 ^a ± 0.00	0.01 ^a ± 0.00	0.01 ^a ± 0.00
	<i>LC</i> 2607	ND ²	ND ²	ND ²	0.03 ^a ± 0.00	0.01 ^a ± 0.00
Sum total	<i>LC</i> ASCC290	46.06 ^a ± 0.01	37.02 ^b ± 0.13	39.07 ^b ± 0.15	39.60 ^b ± 0.12	37.35 ^b ± 0.05
	<i>LC</i> 2607	46.01 ^a ± 0.15	37.54 ^b ± 0.01	36.44 ^b ± 0.11	38.38 ^b ± 0.09	36.89 ^b ± 0.11

Results expressed as mean ± standard error (SE) in ng/μL of fermented soymilk (n = 6). Statistical analysis by means of one-way ANOVA.

a,b Means in the same row with different lowercase scripts are significantly different (P < 0.01).

¹Initial concentration – is the concentration of isoflavones at peak β-glucosidase activity corresponding with maximum biotransformation of the isoflavones.

²ND - Not detected.

*Significant (P<0.01).

LC – *Lactobacillus casei*.

ASCC – Australian Starter Culture Centre.

Table 7.2 First order kinetic parameters for degradation of soy isoflavones in fermented soymilk using *Lactobacillus casei* ASCC 290 and *Lactobacillus casei* 2607 during storage at -80°C, 4°C, 25°C and 37°C

Isoflavone compounds	Storage temperature (°C)	Rate constants ($\kappa \times 10^2 \text{ day}^{-1}$)	
		<i>Lactobacillus casei</i> ASCC290	<i>Lactobacillus casei</i> 2607
Glycitin	-80	1.79	1.48
	4	1.30	1.46
	25	1.28	1.22
	37	1.23	0.79
Daidzin	-80	0.93	2.54
	4	0.84	1.77
	25	0.84	1.61
	37	0.79	1.46
Genistin	-80	1.60	2.14
	4	1.65	2.16
	25	2.25	2.31
	37	2.35	2.33
Glycitein	-80	0.48	1.05
	4	0.55	1.21
	25	0.78	1.39
	37	0.90	1.44
Daidzein	-80	0.13	0.08
	4	0.14	0.09
	25	0.16	0.09
	37	0.17	0.10
Genistein	-80	0.33	0.11
	4	0.33	0.15
	25	0.36	0.17
	37	0.35	0.31
Total isoflavones	-80	0.41	0.33
	4	0.40	0.38
	25	0.45	0.37
	37	0.45	0.45

¹Rate constant (κ) – is the amount of isoflavone losses in ng/ μ L of soymilk per day (day^{-1}) at a specific storage temperature

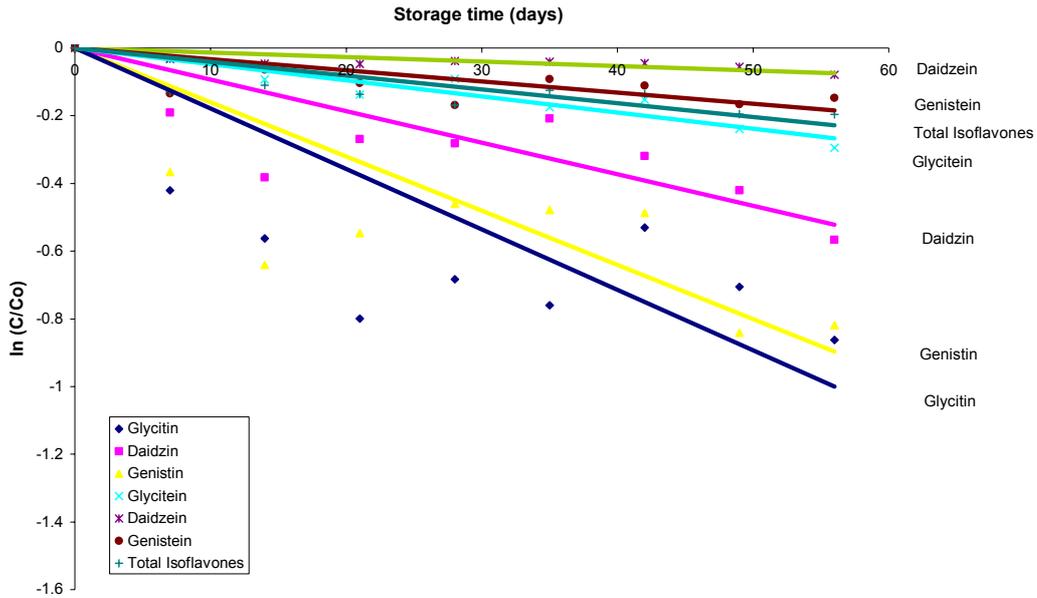


Figure 7.1 Isoflavone degradation in fermented soymilk using *L. casei* ASCC 290 during storage at -80°C . $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone compound to its initial concentration at the specified storage temperature

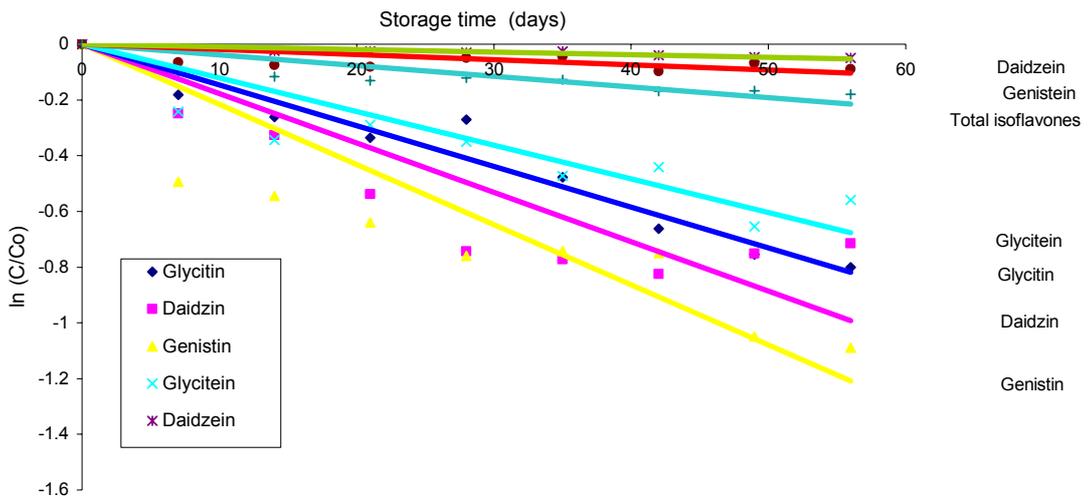


Figure 7.2 Isoflavone degradation in fermented soymilk using *L. casei* 2607 during storage at 4°C. $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone isomer to its initial concentration at the specified storage temperature

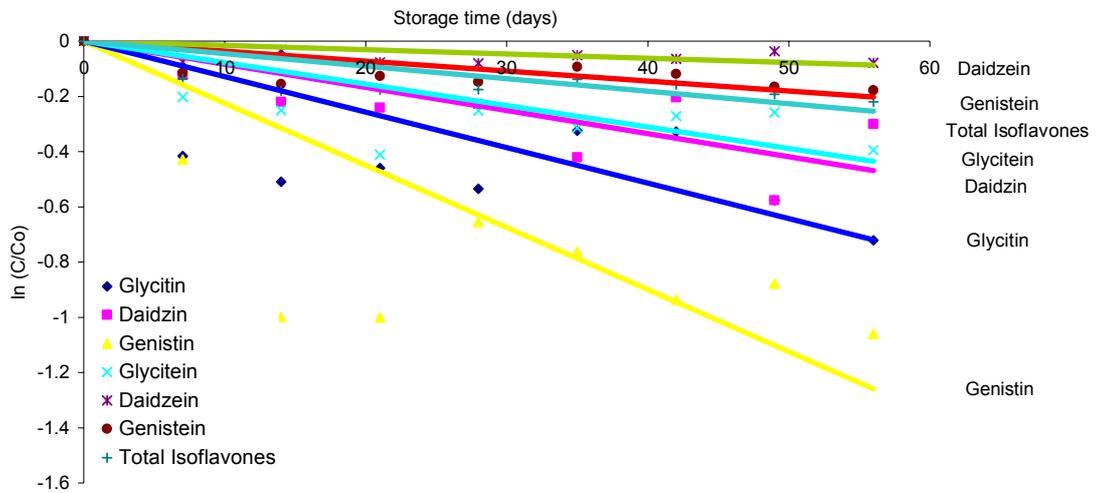


Figure 7.3 Isoflavone degradation in fermented soymilk using *L. casei* ASCC 290 during storage at 25°C. $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone isomer to its initial concentration at the specified storage temperature

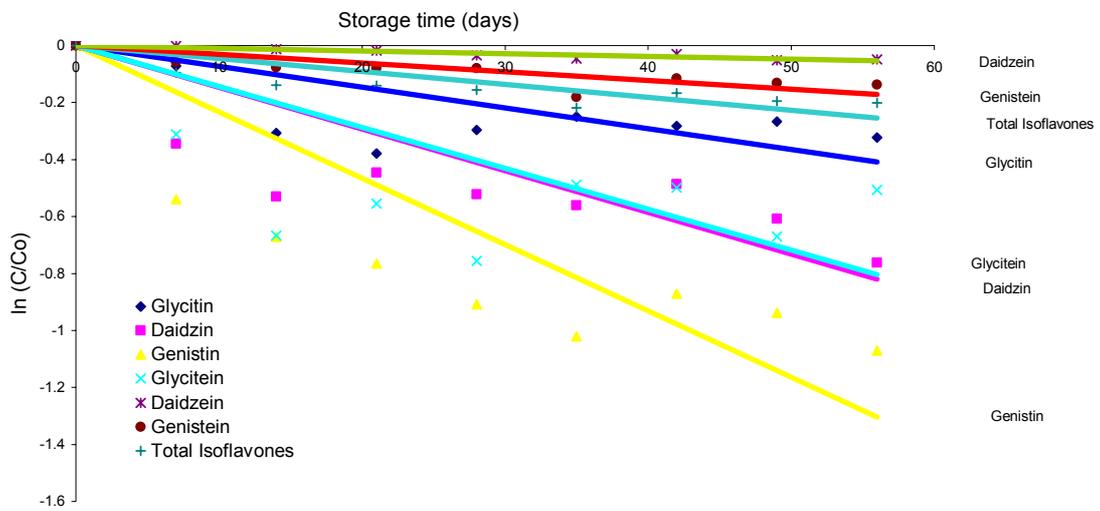


Figure 7.4 Isoflavone degradation in fermented soymilk using *L. casei* 2607 during storage at 37°C. $\ln(C/C_0)$ is the natural logarithm of the ratio of the concentration of the isoflavone isomer to its initial concentration at the specified storage temperature

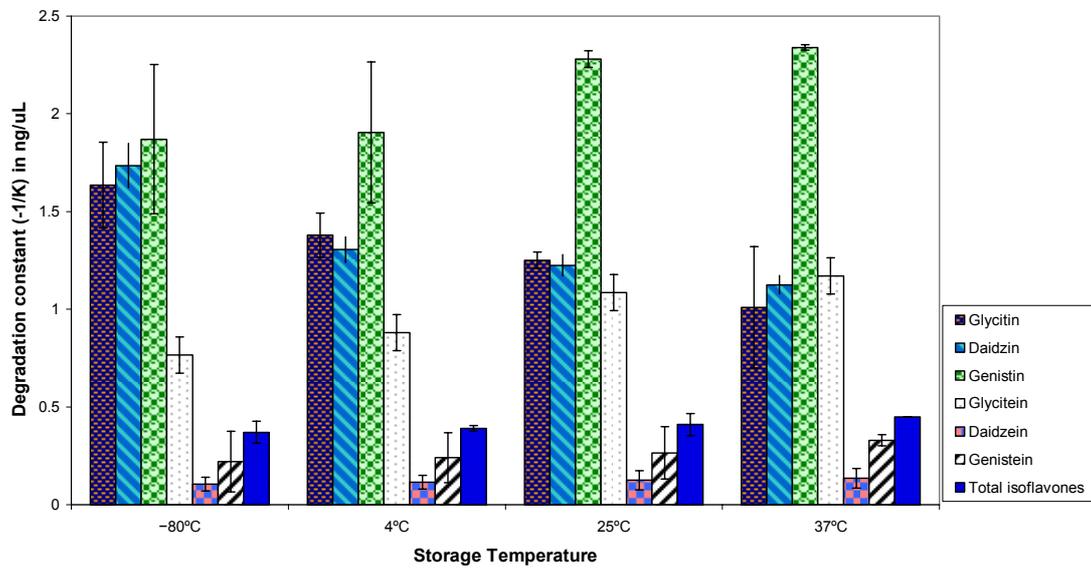


Figure 7.5 Effect of storage temperature on the degradation of isoflavones in fermented soymilk. Degradation constant ($-1/\kappa$) is the rate of degradation of the isoflavone compound in fermented soymilk at the specified storage temperature

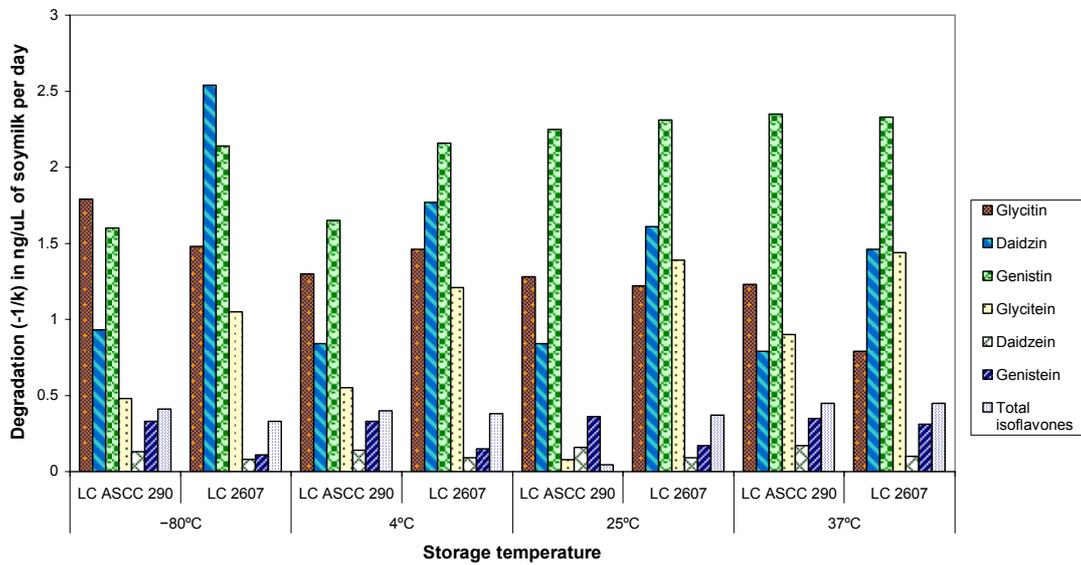


Figure 7.6 Comparison of the influence of the two *L. casei* strains on the degradation of isoflavones in fermented soymilk during storage at different temperatures. Degradation constant ($-1/\kappa$) is the rate of degradation of the isoflavone compound in fermented soymilk at the specified storage temperature

8.0 Profiling and Quantification of Isoflavones in Soymilk from Soy Protein Isolate using Extracted Ion Chromatography and Positive Ion Fragmentation Techniques

8.1 INTRODUCTION

Isoflavones as a class of isoflavonoids are distinct in their structure, sources and activities which have come under increasing scrutiny due to their estrogen-like properties (Kurzer, 2000). They have polyphenolic structure in which the B ring is attached at carbon 3 position of phenolic C ring instead of the more typical carbon 2 position as in most flavonoids (Figure 8.1) and are found in the greatest amounts in soybeans among legumes consumed by humans. They have been reported to prevent the oxidation of low-density lipoprotein (LDL), thus reducing atherogenesis, to decrease bone reabsorption in a manner similar to the activity of estrogen (Gao, & Yamaguchi, 1999), to lower the prevalence of breast and prostate cancers in societies where consumption of soy is common (Watson *et al.* 2000), and to reduce the risk of atherosclerosis (de Kleijn *et al.* 2002), neuro-degeneration (Kim *et al.* 2000) and osteoporosis (Cheichi *et al.* 2002). The extent and nature of isoflavone glycoside configuration whether aglycone, simple glycoside, malonyl-, or acetylglycoside, may influence bioavailability upon consumption (King *et al.* 1996). Thus it is important to know the total amount of each of the isoflavone compounds in a food, and the relative proportions of the bioactive aglycone components, daidzein, genistein and glycitein and their various glycosides (King & Bignell, 2000). A number of studies have shown that the isoflavone content of soybean is strongly influenced by the genetic and environmental factors (Tsukamoto *et al.* 1995). In addition, preparation of soy protein isolate, while important in the removal of flatulence factors and beany flavours (Rackis, 1979), is known to substantially reduce the amount of total isoflavones (upto 53%) through extraction process using organic solvents (Wang, & Murphy, 1996). The intake level of isoflavones required to provide an optimal health

benefit is still not known. However, intake of soy food products in daily diets in such countries as China and Japan, has been estimated to average about 30 to 40 mg per day (Chen, *et al.* 1999; Wakai *et al.* 1999), expressed as aglycone equivalent. Interestingly, there is low mortality from a number of cancers and from cardiovascular diseases in these countries. This contrasts with estimates for the USA of approximately 0.1 mg per day (Strom *et al.* 1999), which would also be expected to apply to the entire western world including Australia. There are very few published values for the isoflavone content of Australian soy foods (Knight *et al.* 1998; Dalais *et al.* 1997). Only one report (Knight *et al.* 1998) listed daidzein and genistein contents in soy milk and soy infant formula purchased from Australian supermarkets. Values for total isoflavone content of soy milk and other soy based foods such as soy bread, soy flour, tofu and soy sauce in Australia have been reported by King & Bignell (2000). Probiotic micro-organisms including *Lactobacillus* and *Bifidobacterium* have been known to possess endogenous β -glucosidases which can play an important role in altering the profile of isoflavones during fermentation (Chapter 3.0). Although each group of probiotics has varying potential in the hydrolysis of isoflavones during fermentation (Chapter 4.0), the hydrolytic action has been found to cause major increases in the concentration of bioactive isoflavone aglycones and concomitant decrease in the concentration of isoflavone glycosides (Chapters 4.0, 5.0 and 6.0).

It is critical that the isoflavone content and their concentration in soy protein isolate are determined as it is the major ingredient in the preparation of unfermented and fermented soymilk. Accordingly, there is a need to develop a method for the rapid and accurate identification and quantification of these isoflavonoids. Most methods for the analysis of isoflavones are based on high performance liquid chromatography (HPLC)- or capillary electrophoresis (CE) separation with uv-, fluorescence- or electrochemical detection (Chen *et al.* 2001; Wang *et al.* 2002). These methods however are limited to the detection of a limited number of known compounds and are not applicable for the characterisation of unknown isoflavonoids in a crude mixture (Prasain *et al.* 2003). Mass spectrometry (MS) combined with LC-MS/MS is currently the most sensitive and

selective analytical method for the rapid qualitative and quantitative analysis of known compounds as well as for the identification of unknown compounds from purified samples of natural products (Barnes *et al.* 1994; Prasain *et al.* 2002). Its unique ability to filter and isolate molecular ions with specific mass-to-charge (m/z) ratios from a complex mixture makes MS a valuable tool for analysis. Generally, electrospray ionization mass spectrometry (ESI-MS) provides a mass spectrum with little or no fragmentation, and this technique is suitable for the characterization of a single compound as well as complex mixture of natural products. We report an integrated approach consisting of LC-MS/MS, including neutral ion-scan mass spectrometry for the quantification and identification of isoflavones in fermented and unfermented soymilk made from soy protein isolate (SPI; SUPRO 590). To the best of our knowledge, we for the first time also report an intricate link between the occurrence of isoflavone aglycones and specific aldehydes in soymilk.

8.2 MATERIALS AND METHODS

8.2.1 Bacteria

Pure culture of *B. animalis* ssp. *lactis* Bb12 was obtained from Chr. Hansen Pty. Ltd. (Bayswater, Vic, Australia) and the purity was confirmed using the procedure in section 3.2.1. The stock culture was then propagated and stored at -80°C in 40% glycerol.

8.2.2 Bacterial growth media

Rehydrated de Mann Rogosa Sharpe (MRS) broth (pH adjusted to 6.7 using 5.0 M sodium hydroxide) (de Mann *et al.* 1960) was prepared according to manufacturer instructions (Oxoid Ltd., West Heidelberg, Vic, Australia) and autoclaved at 121°C for 15 min.

8.2.3 Soymilk manufacture

Soy protein isolate (SPI; SUPRO 590), supplied by The Solae Co. (Chatswood, NSW, Australia), was used in the production of soymilk at 40 g per litre according to resulting into a homogenised milk-like product devoid of any protein suspensions as mentioned in section 3.2.2. After reconstitution, the soymilk was dispensed into a glass bottle at 250 mL, then autoclaved at 121°C for 15 min. After cooling to room temperature, the pH was adjusted in a laminar flow to 6.7 using 5 M sodium hydroxide.

8.2.4 Fermentation of soymilk with *B. animalis* ssp. *lactis* Bb12

The micro-organism was activated in MRS broth at 37°C for 20 h successively 3 times followed by a fourth activation in sterile soymilk as outlined in section 3.2.3. In order to conduct studies in the changing profile and concentration of isoflavones, a 250 mL of sterile soymilk was inoculated (in duplicate) with the active culture (5% v/v), and incubated at 37°C for 24 h to achieve peak β -glucosidase activity thus transforming the predominant isoflavone glycosides to the aglycone forms as found in our earlier study (Chapter 4.0). Aliquots of 50 mL were withdrawn aseptically at 12 h intervals and then stored immediately at -80°C for analysis of isoflavones. The frozen aliquot was freeze-dried using a Dynavac® FD300 freeze drier (Rowville, Vic, Australia) for extraction and analysis of isoflavone using reverse-phase HPLC and liquid chromatography mass spectrometry (LC - MS).

8.2.5 Extraction of isoflavones for LC-MS/MS analysis

The extraction of isoflavones, including malonyl-, acetyl-, and β -glycosides, and aglycones from fermented and non-fermented soymilk was performed in triplicate using a modified version of a method described by Tsangalis *et al.* (2002). A 1-g freeze-dried sample was added to 50 mL of methanol in a 150 mL round bottom flask and refluxed on a heating mantle for 1 h. The mixture was then filtered through a Whatman No. 1 filter paper into a 100 mL volumetric flask. The

remaining dried soy matter was washed with the filtered portion and then refiltered into the same flask. A 5 mL aliquot was taken and after adding 60 μ L of internal standard (ISTD) flavone solution (10 mg/50 mL), the sample was dried under a stream of nitrogen using a Techne sample concentrator (Pearce Biotechnology Inc., Rockford, IL, USA). The resultant dried matter was then resuspended in 1 mL of 10 mM ammonium acetate buffer (containing 0.1% trifluoro-acetic acid) and methanol (50:50) solution and centrifuged ($14,000 \times g$ for 30 min) using an Eppendorf centrifuge (model 5415C; Crown Scientific Pty. Ltd., Vic, Australia), then filtered through a 0.5 - μ m FH membrane prior to transferring to HPLC vials.

8.2.6 Isoflavone standards

All the aglycone standards of genistein, daidzein and glycitein as well as flavone (ISTD) were purchased from Sigma Chemicals (Castle Hill, NSW, Australia) while the β -glycoside standards of genistin, daidzin, puerarin and glycitin as well as daidzein metabolite equol were purchased from Indofine Chemical Co. (Sommerville, NJ, USA). Genistein, genistin, flavone, daidzein, and equol were prepared in HPLC grade methanol, and daidzin, glycitein and glycitin in ethanol due to their varied solubility characteristics.

8.2.7 LC-MS and LC-MS/MS instrumentation

Components of the isoflavone extract were separated by HPLC using a 150 mm \times 2.1 mm internal diameter (i.d), C18 aquapore reversed-phase column pre-equilibrated using a gradient elution system with 0.05% methanol aqueous solution and 0.05% formic acid aqueous solution. The mobile phase was initially composed of 10:90 acetonitrile/water (both containing 0.05% methanol and formic acid), followed by a linear gradient to 40% methanol over 30 min at a flow rate of 0.95 mL/min. The column eluate was passed into the ionspray ionization interface operating in the positive mode of a PE Sciex (Concord, ON, Canada) API III triple-quadrupole mass spectrometer. The voltage on the ionspray interphase was 121 V, and the orifice potential

was set at 40 V. Positive ion mass spectra were recorded over an m/z range of 200-600. Selected $[M + H]^+$ were analysed by collision-induced dissociation with 90% argon/ 10% nitrogen and the daughter ion mass spectra were recorded. Neutral loss scanning (a tandem mass spectrometric mode to obtain an array of all parent ions that lose a common neutral fragment) of the isoflavone extract were acquired in the positive ion mode with a dwell time of 5 ms and a step size of 1 m/z . The MS/MS analyses of isoflavonoids glycosides were performed using a Q-TOF mass spectrometer (Micromass, Manchester, U.K.) equipped with an electrospray ion source. Product ion spectra were obtained in the positive and negative ion modes. Product ion spectra were obtained by selecting the protonated and deprotonated ions for collision (energy = 40 eV), using argon as a collision gas.

8.2.8 Statistical analysis

To find the difference in isoflavone concentrations in soymilk before and after 12 h fermentation, means were analysed using one-way analysis of variance (ANOVA) and 99% confidence levels using Microsoft[®] Excel Statpro[®] as described by Albright *et al.* (1999). ANOVA data with a $P < 0.01$ was classified as statistically significant.

8.3 RESULTS AND DISCUSSION

8.3.1 Compositional variations of isoflavones during fermentation

The profiles of isoflavone glycosides and aglycones in unfermented soymilk ($h = 0$) is shown in Figure 8.2 while that after 12 h of fermentation at 37°C using *B. animalis* ssp. *lactis* Bb12 is shown in Figure 8.3. We reported earlier (Chapter 4.0) that unfermented soymilk contained higher amounts of isoflavone glycosides than the aglycone components. During fermentation, there was an increase in the aglycone concentration with a concomitant reduction of the isoflavone glycosides. The concentration of isoflavones compounds was influenced by the β -

glucosidase activity and the growth of the micro-organism in the soymilk (Table 3.2). The increase in isoflavone aglycones was due to the hydrolytic ability of endogenous β -glucosidases (Chapter 4.0) and possibly by the crude β -galactosidase enzymes within the bacterial cell-wall. It is interesting to note that 11 of the 13 known isoflavones in soymilk were detected in the soymilk made from SPI (SUPRO 590). Malonylglycitin and equol were not detected in the unfermented and fermented soymilk as shown in Figures 8.2 and 8.3, respectively. According to Wang and Murphy (1994b) soybeans have variable isoflavone contents depending on the variety and environmental conditions (location and/or crop year). Equol is a daidzein metabolite and occurs mostly as a result of enzyme induced reduction in the intestinal tract. Equol may never be detected in an *in vitro* system, but is detectable from physiological fluids such as blood plasma and urine using most analytical techniques including HPLC and LC – MS (King, & Bursil, 1998; Jenkins *et al.* 2002). This was confirmed using several analytical procedures of soy isoflavones in unfermented and fermented soymilk (Chapters 4.0 to 7.0).

8.3.2 Identification and quantitation of isoflavone compounds

The peak heights in Figures 8.2 and 8.3 show the intensity or the concentration of each compound in the soymilk. Peak 3 (EIC 433) representing glycoside genistin was the tallest peak followed by peak 1 (EIC 417) representing glycoside daidzin in Figure 8.2. The malonyl forms, that is, peaks 4 (EIC 503) and 6 (EIC 519) represented by malonyl-daidzin and malonyl-genistin, respectively, were the least in concentration in unfermented soymilk as compared three other classes of isoflavones. Acetyl-genistin (peak 9, EIC 475) was the highest in concentration amongst the acetylated forms (Figure 8.2). Unlike the malonyl forms, there were three acetylated isoflavones detected in the soymilk with the other two being peaks 5 (EIC 459) and 7 (EIC 489) represented by acetyldaidzin and acetylglycitin, respectively. In general, isoflavone glycosides (genistin, daidzin and glycitin) were the most predominant forms followed by the aglycones. In unfermented soymilk, the major peaks were without question those of the isoflavone glycosides

such as daidzin (peak 1) and genistin (peak 3). Acetyldaidzin (peak 5) also appeared to be in high concentration relative to the rest of the isoflavones. Interestingly, aglycone daidzein (peak 8) also occurred in relatively high concentration in the soymilk before fermentation.

There was a noticeable shift in the concentration of the classes of isoflavones in the soymilk after 12 h of fermentation with probiotic *B. animalis* ssp. *lactis* Bb12 as shown in Figure 8.3. The breaking down of β -1, 6 glycoside bonds in the isoflavone glycosides was due to the activity of endogenous β -glucosidase enzyme and could be in part due to the presence and activity of crude β -galactosidase enzyme (Tochikura *et al.* 1986). The products of hydrolysis were the sugar moiety as well as the aglycones in the soymilk which provided a source of nutrients for the growth of the micro-organism (Ballongue, 1993). As shown, there was a reduction in the concentration of isoflavone glycosides of peaks 1, 2 and 3 represented by daidzin (EIC 417), glycitin (EIC 447), and genistin (EIC 433), respectively. In contrast, there was a considerable increment in the intensity of aglycones, daidzein, glycitein and genistein represented by peaks 8 (EIC 255), 10 (EIC 285) and 11 (EIC 271), respectively. An important change in the isoflavone profile in the soymilk during fermentation was the occurrence of major peaks (with higher concentration) of mainly aglycones. The highest peaks were those of 11, 8 and 10 representing aglycones genistein, daidzein and glycitein, respectively. Genistein, daidzein and glycitein increased in their intensities after 12 h fermentation (Figure 8.3) to 4.7×10^7 , 4.2×10^7 , and 1.8×10^7 from 0.75×10^7 , 1.2×10^7 and 0.375×10^7 , respectively before fermentation (Figure 8.2) in the soymilk. The increment of aglycone components during fermentation is important in enhancing the biological activity of the soymilk due to their structural similarity to the human estrogen. This structural similarity to human estrogen enables their possession of varying estrogen-like activities. Genistein has been found to be the most estrogenically potent aglycone followed by daidzein and glycitein (Valachovicova *et al.* 2004).

Table 8.1 shows the quantitation of isoflavones and the changes in concentration of glycosides and aglycones occurring during fermentation with *B. animalis* ssp. *lactis* Bb12. Using LC-MS,

the total amount of isoflavones detected in the soymilk was about 56.08 µg/mL. The isoflavone profile consisted predominantly of upto 84.8% glycosides before fermentation. However, after 12 h of fermentation, the total glycoside concentration reduced by a factor of 4.95 times than the initial concentration. Concomitantly, the amount of isoflavone aglycones instead increased by a factor of 5.46 times than the initial concentration due to the hydrolytic action of crude endogenous β-glucosidase and β-galactosidase within the microbial cell wall.

8.3.3 Separation of existing isoflavone compounds in fermented and unfermented soymilk

Based on molecular weight and polarity of the isoflavone compounds in the soymilk, separation occurred in the stationary phase of the column. High performance liquid chromatography-mass spectrometry (HPLC-MS) offers the distinct advantage of identification of compounds based on molecular weight and ion charge separation. The degree of polarisation of two out of eleven compounds could not allow for a clear separation of the isoflavones which were identified as malonylgenistin (EIC 519) and acetylglycitin (EIC 489) (Figure 8.4). As a result of the near equal polarity, they appeared to have similar charges and consequently near equal affinity to the stationary phase of the C₁₈ column used. In our previous studies, the use of ammonium acetate plus trifluoroacetic acid and acetonitrile in the mobile phase rather than formic acid gave better separation of the two compounds (manonyl genistin and acetyl glycitin), although we had encountered difficulties in the separation of genistein and equol (Chapter 4.0 and Chapter 5.0). The two compounds appear as peaks 6 and 7, respectively (Figures 8.2 and 8.3). Figure 4 shows the enlarged manifestation of the protonated peaks of the two compounds. Using LC-MS/MS, the protonated ions of the isoflavone compounds enabled their identification despite the inability to clearly separate them.

8.3.4 Product ion analysis of isoflavone glycosides in soymilk

To understand the mass spectrometric behaviour of isoflavones, LC-MS analysis of authentic daidzin, genistin and glycitin was performed using ESI in the positive ion mode. The molecular mass and structural and elemental formulae of these glycosides were shown in Figure 8.5. Product ion spectra of isoflavone glycosides, glycitin, genistin and daidzin represented by A, B and C respectively (Figure 8.5) revealed several diagnostic product ions. There are similarities and differences in the fragmentation patterns of the three isoflavone glycosides. An important similarity was the loss of 18 Da from the parent peaks of m/z 447 to 428, 433 to 415 and 417 to 399 for glycitin, genistin and daidzin, respectively, representing the neutral loss of water (H_2O). Peaks of m/z 417 and 433 appear as parent peaks in daidzin (A) and genistin (B) respectively. The other similarity was the occurrence of respective protonated aglycone ions in each mass spectrum of all three respective glycosides represented by a loss of 162 Da. The loss of ions from the parent peaks of an isoflavone glycoside leading to emergence of a protonated Y_o^+ aglycone is associated with the loss of an entire sugar unit. The occurrence of the respective Y_o^+ aglycone ions at m/z 285, 271 and 255 in A, B and C, respectively is a further confirmation of the identity of the isoflavone glycosides determined in the soymilk. The fragmentation pathway of any molecule is therefore unequivocal confirmation of the uniqueness of the molecule. For instance isomeric compounds like puerarin and diadzin, both of equal molecular weight of 416 and of almost similar molecular structure (Figure 8.6) can be confirmed to be different using LC-MS/MS positive ion fragmentation. On the other hand, (B) in positive mode showed fragments of ions at m/z 399, 255 and 205, puerarin (A) showed two prominent diagnostic ions of m/z 297 and 267, respectively indicating neutral losses of 120 and 150 Da, respectively. The loss of 120 Da was indicative of the occurrence of C-glycosides (Waridel *et al.* 2001). A series of ions at m/z 381 and 363 in puerarin were obtained due to the successive neutral losses of water molecules. Two types of typical isoflavone product ions existed namely C- and O-glycosides. By definition, C-glycosides are compounds in which the inter-glycosidic oxygen atom has been replaced by a

carbon atom to produce a stable glycoside derivative that is not prone to enzymatic or chemical hydrolysis (Postema *et al.* 2005). To be suitable mimic of the *O*-glycoside, *C*-glycoside must possess conformation similar to that of the parent *O*-glycoside or adopt that of the parent *O*-glycoside conformation that still elicits a biological response (Postema *et al.* 2005). The *O*-glycosides have sugar substituents bound to a 7-hydroxy group of the aglycone, whereas *C*-glycosides have sugar substituents bound to a carbon of the aglycone, generally at positions C-6 and C-8. Several studies (Wei *et al.* 1995; Wang *et al.* 1998; Tsuruta, *et al.* 1999) have shown that the substitution of the inter-glycosidic oxygen atom with a carbon atom did not greatly alter biological activity. Glycosides such as glycitin, genistin and glycitin undergo facile losses of the sugar moiety on MS-MS fragmentation and this has been attributed to the weaker C-O bond between the sugar and the aglycone (Prasain *et al.* 2003). Puerarin did not show similar pattern as that of daidzin due to the C-C bond linking the sugar and the aglycone (Figure 8.6).

Figure 8.7 showed the comparison of the product ions obtained in ESI-MS/MS of the isoflavone aglycones, daidzein (D), genistein (E) and glycitein (F) when subjected to positive ion fragmentation on MS/MS. Glycitein (F) showed a series of ions beginning with parent peak at m/z 285, 270, 257, 229, 196 and 166 represented by losses of 15 Da, 13 Da, 28 Da, 32 Da and 30 Da, respectively. The radicals represented by the successive losses during glycitein fragmentation are methyl (CH₃), carbon hydride (CH), carbon monoxide (CO), methanol (CH₃OH) and a methanal (HCHO). Aglycone genistein (E) on the other hand showed a series of ions at m/z 271, 243, 215, 187 and 153 represented by three successive losses of 28 Da and 34 Da. The fragmentation pattern of genistein therefore involved 3 successive losses of carbon monoxide (CO), followed by ethanal (CH₃CHO). Daidzein (D) on fragmentation showed ions at m/z 255, 227, 199, and 145 represented by 2 successive losses of 28 Da, followed by an unidentifiable radical of 54 Da. These unidentifiable compounds could possibly be known using nuclear magnetic resonance (NMR) techniques. Ions fragmented from daidzein were a representation of carbon monoxide (CO) radical and then a pentanal (CH₃CH₂CHO), respectively. It was

interesting to observe the occurrence of at least a carbon monoxide (CO) and an aldehyde (pentanal, ethanal and a methanal, for daidzein, genistein and glycitein, respectively) as part of the ions spectra. Aldehydes such as pentanal and hexanal have been known to occur in soy based matrices (Tsangalis, & Shah, 2004) and act as a prebiotic to support the growth of probiotic micro-organisms in the soymilk. Accordingly, SPI with higher oligosaccharide content also had higher levels of pentanal and hexanal. The occurrence and variations in the levels of pentanal and hexanal in SPI could be influenced by the extent of formation of these volatiles in the defatted soy flour from which SPI is prepared. Fujimaki *et al.* (1965) reported that hexanal and some other volatile components were formed by simple autoxidation of lipids remaining in defatted soybean. Thus the occurrence of aldehydes during fragmentation of isoflavone aglycones could be due to autoxidative process in lipids but the link of specific aldehydes to specific aglycone parent ions on fragmentation remains to be investigated further. According to Tsangalis & Shah (2004), fermentation of soymilk with strains of *Bifidobacterium* also decreased the levels of hexanal and pentanal. Thus it appears that the aldehydes could be metabolised for growth and viability of micro-organisms in soymilk which in the process causes biotransformation of isoflavone glycosides.

8.3.5 Comparison of product ions obtained in ESI-MS/MS of the malonyl-, and acetylglycosides detected in soymilk

The analysis of the ion spectra also involved that of malonyl and acetyl-glycosides in the soymilk and is shown in Figure 8.8. Malonyl-glycitin was not detected in the soymilk. There was no commonality in the fragmentation pattern of the acetyl- and malonyl-glycosides except the non successive neutral loss of 18 Da represented by water (H₂O) in all the glycosides. However, the occurrences of respective Y₀⁺ aglycone ions in the fragment spectrum of each of the acetyl- and malonyl-glycoside were detected. For example, the aglycone ions of daizein (*m/z* 255), genistein (*m/z* 271) and glycitein (*m/z* 285) appeared on the product ion spectra of acetyl - and

malonyldaidzin (G & J), acetyl - and malonylgenistin (H & K), and acetyl-glycitin (I), respectively. Interestingly, there was no occurrence of respective main glycoside ions such as genistin, daidzin and glycitin in the spectra of fragmented acetyl- and malonyl-glycosides. A summary of product ions and molecules formed during ESI-LC-MS fragmentation of isoflavones in soymilk is shown in Table 8.2 and a summary of protonated fragments from each isoflavone detected is shown in Table 8.3.

8.4 CONCLUSIONS

Since there was no similarity in the fragment product ions, this indicates the uniqueness of each isoflavone compound. Although there were differences in the spectra of product ions from individual isoflavones in the soymilk, the fragmentation patterns of classes of isoflavone i.e. aglycones, glycosides, acetyl-, and malonyl- forms followed a similar trend. There was no similarity of particular peaks amongst the isoflavone aglycones but there was a similarity in the trend of loss of carbon monoxide, at least successively with daidzein and genistein. All isoflavone flavone glycosides including the malonyl and acetyl forms detected in the soymilk had respective aglycone ions as major peaks in the spectra. Glycosides such as daidzin and glycitin had a similar fragment ion of m/z 206 while genistin, acetylgenistin, acetylglycitin, malonyldaidzin and malonylgenistin had a common occurrence of fragment ion of 184 m/z ($C_{12}H_8S$). Even though there were similarities in the fragments amongst the isoflavone glycosides and in the trend of losses ions and molecules during fragmentation of each class of isoflavones, each compound however, had a unique fragmentation pattern leading to their unequivocal identification. The identification of isoflavones in fermented and unfermented soymilk is necessary to generate information for *in vivo* and *in vitro* studies and for the authentication of isoflavones in soy based drinks and beverages.

Table 8.1 Concentration of isoflavones ($\mu\text{g/mL}$) in fermented soymilk using *Bifidobacterium animalis* ssp. *lactis* Bb12.

Isoflavone compounds	0 h	6.0 h	12.0 h	P- value
Glycitin	15.95 ^a \pm 1.3	5.31 ^a \pm 0.5	5.56 ^a \pm 0.6	0.0000*
Daidzin	6.57 ^a \pm 0.6	0.87 ^{ab} \pm 0.1	1.11 ^{ab} \pm 0.2	0.0000*
Genistin	20.44 ^a \pm 2.0	1.45 ^b \pm 0.4	1.01 ^b \pm 0.1	0.0000*
β -glycosides ¹	37.13 ^a \pm 2.8	7.63 ^{bc} \pm 0.6	7.68 ^{bc} \pm 0.6	0.0000*
Glycitein	0.37 ^a \pm 0.1	1.48 ^a \pm 0.3	1.58 ^a \pm 0.2	0.0000*
Daidzein	4.15 ^a \pm 1.0	19.39 ^b \pm 0.6	19.04 ^b \pm 0.3	0.0000*
Genistein	3.99 ^a \pm 0.8	25.21 ^{bc} \pm 1.1	25.86 ^{bc} \pm 0.3	0.0000*
Aglycones ¹	8.52 ^a \pm 1.6	46.1 ^{bc} \pm 1.2	46.5 ^{bc} \pm 0.3	0.0000*
Malonylglycitin	ND	ND	ND	N/A
Malonydaidzin	0.99 ^a \pm 0.3	0.23 ^a \pm 0.0	0.15 ^a \pm 0.1	0.0000*
Malonylgenistin	3.02 ^a \pm 0.2	1.15 ^a \pm 0.2	1.10 ^a \pm 0.2	0.0002*
Malonylglycosides ¹	4.01 ^a \pm 0.5	1.38 ^a \pm 0.3	1.25 ^a \pm 0.2	0.0000*
Acetylglycitin	2.25 ^a \pm 1.8	0.46 ^a \pm 0.1	0.35 ^a \pm 0.0	0.0573*
Acetyldaizidin	1.09 ^a \pm 0.2	0.15 ^a \pm 0.1	0.03 ^a \pm 0.0	0.0000*
Acetylgenistin	3.08 ^a \pm 0.3	0.38 ^b \pm 0.2	0.28 ^b \pm 0.1	0.0000*
Acetylglycosides ¹	6.42 ^a \pm 0.9	0.99 ^a \pm 0.6	0.67 ^a \pm 0.1	0.0000*
Equol	ND	ND	ND	N/A
Total Isoflavones ²	56.08 ^a \pm 0	56.08 ^a \pm 0	56.08 ^a \pm 0	0.0000*

Results expressed as mean \pm standard error (SE) in $\mu\text{g/mL}$ of soymilk (n = 6).

Statistical analysis by means of one-way ANOVA.

^{a,b,c} Means in the same row with different lower case scripts are significantly different (P < 0.01).

Table 8.2. Summary of product ion data for protonated isoflavones in soymilk during fragmentation of using ESI- LC MS/MS.

Compound	Parent ion Methyl		Carbon hydride	Carbonmonoxide	Carbonmonoxide	Carbonmonoxide	Methanol	^a Unknown	^b Unknown	Methanal	Water	Carbondioxide	Sugar
	[M+H] ⁺	[M+H-CH ₃] ⁺	[M+H-CH] ⁺	[M+H-CO] ⁺	[M+H-2CO] ⁺	[M+H-3CO] ⁺	[M+H-CH ₃ OH] ⁺	Compound	Compound	[M+H-HCHO] ⁺	[M+H-H ₂ O] ⁺	[M+H-CO ₂] ⁺	[M+H-S ₀] ⁺
Daidzein	255			227	199				145				
Genistein	271			243	215	187		153					
Glycitein	285	270	257	229			196			166			
Daidzin	417										399		255
Genistin	433										415		271
Glycitin	447										428		285
Acetyldaidzin	459										441		255
Acetylgenistin	475											431	271
Acetylglycitin	489										471		285
Malonyldaidzin	503										485		255
Malonylgenistin	519										501		271

^aUnknown Compound - Fragment ion of 34 Da from isoflavone genitein. Could be identified using nuclear magnetic resonance (NMR).

^bUnknown Compound - Fragment ion of 54 Da from isoflavone daidzein. Could be identified further using nuclear magnetic resonance (NMR).

Parent ion - The initial peak ion being subjected to MS/MS fragmentation

Table 8.3 Summary of the *m/z*, peaks of fragments and the molecular weights of isoflavones in the soymilk using ESI-MS/MS on positive fragmentation

^a Isoflavones Identity	MW	MS	MS/MS fragments
Daidzein	254	255	227, 199, 137, 93
Genistein	270	271	243, 215, 187, 153, 103
Glycitein	284	285	270, 257, 229, 197, 167, 145, 124, 109, 95
Daidzin	416	417	399, 319, 288, 255, 206, 119
Genistin	432	433	415, 375, 313, 271, 184, 133
Glycitin	446	447	429, 411, 389, 349, 313, 285, 259, 206, 175, 133
Acetyldaidzin	458	459	441, 359, 322, 255, 210
Acetylgenistin	474	475	431, 375, 339, 271, 246, 184
Acetylglycitin	488	489	471, 445, 353, 317, 285, 217, 184, 159
Malonyldaidzin	502	503	485, 443, 405, 329, 287, 255, 233, 184
Malonylgenistin	518	519	500, 459, 315, 271, 227, 184

^aIsoflavone identity – Identification were aided by revelation of molecular weights and fragments from LC-MS/MS and is the list of isoflavones in order of molecular weights detected in the soymilk made from soy protein isolate SUPRO 590.

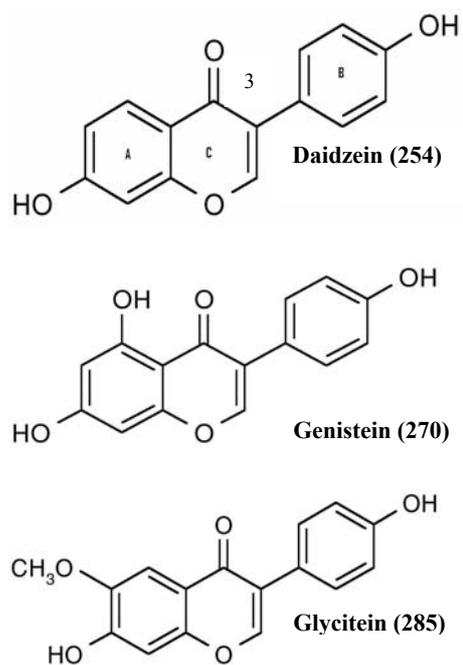


Figure 8.1 Structures of isoflavone aglycones daidzein, genistein and glycitein showing the B ring attachment at carbon 3.

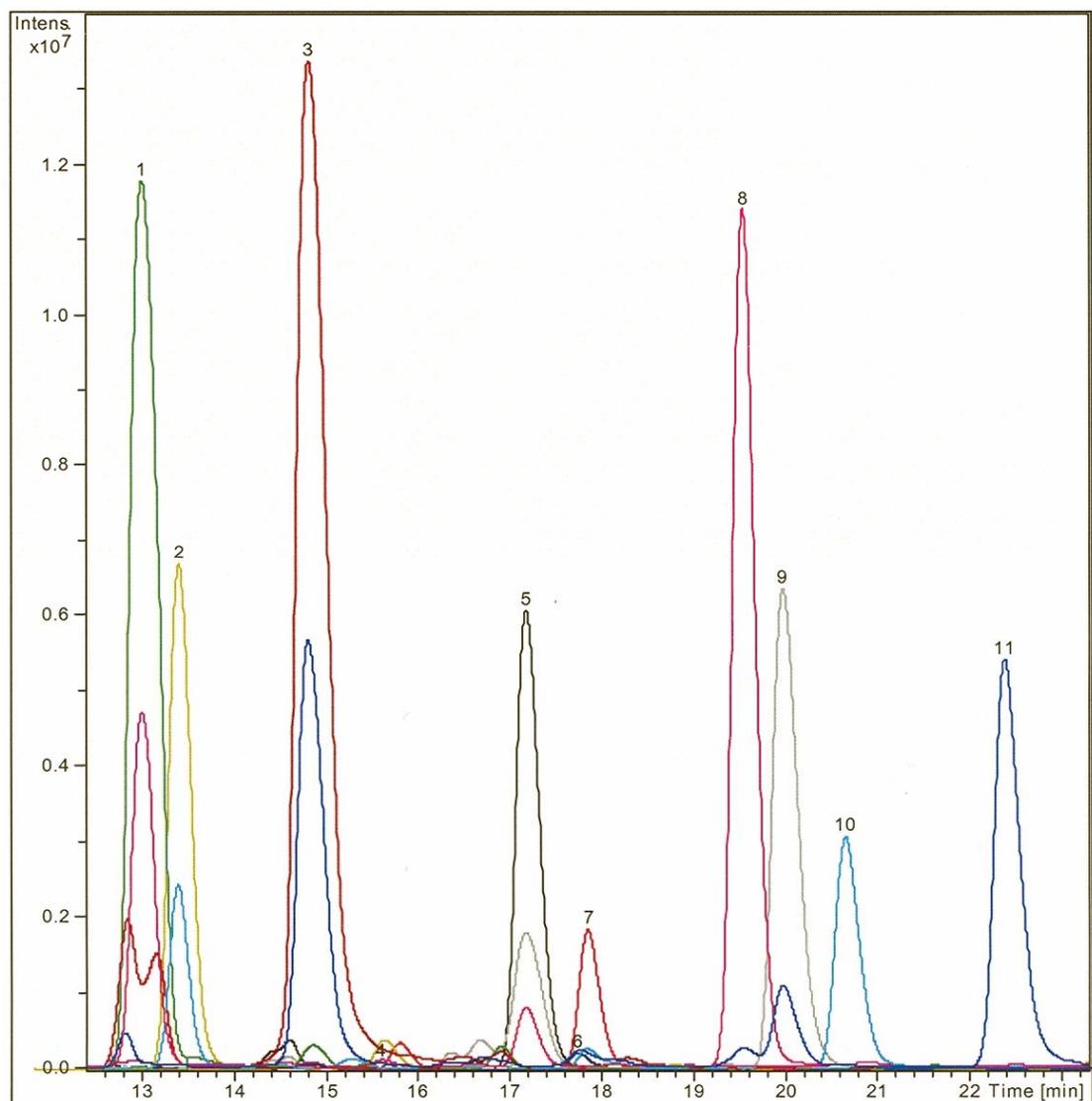


Figure 8.2 Electro-spray ionisation chromatogram using LC-MS of isoflavones in unfermented soymilk (0 h) made from soy protein isolate (SPI; SUPRO 590).

Elution order and peak identification	
1.	Daidzin (EIC; 417)
2.	Glycitin (EIC; 447)
3.	Genistin (EIC; 433)
4.	Malonyldaidzin (EIC; 503)
5.	Acetyldaidzin (EIC; 459)
6.	Malonylgenistin (EIC; 519)
7.	Acetylglycitin (EIC; 489)
8.	Daidzein (EIC; 255)
9.	Acetylgenistin (EIC; 475)
10.	Glycitein (EIC; 285)
11.	Genistein (EIC; 271)

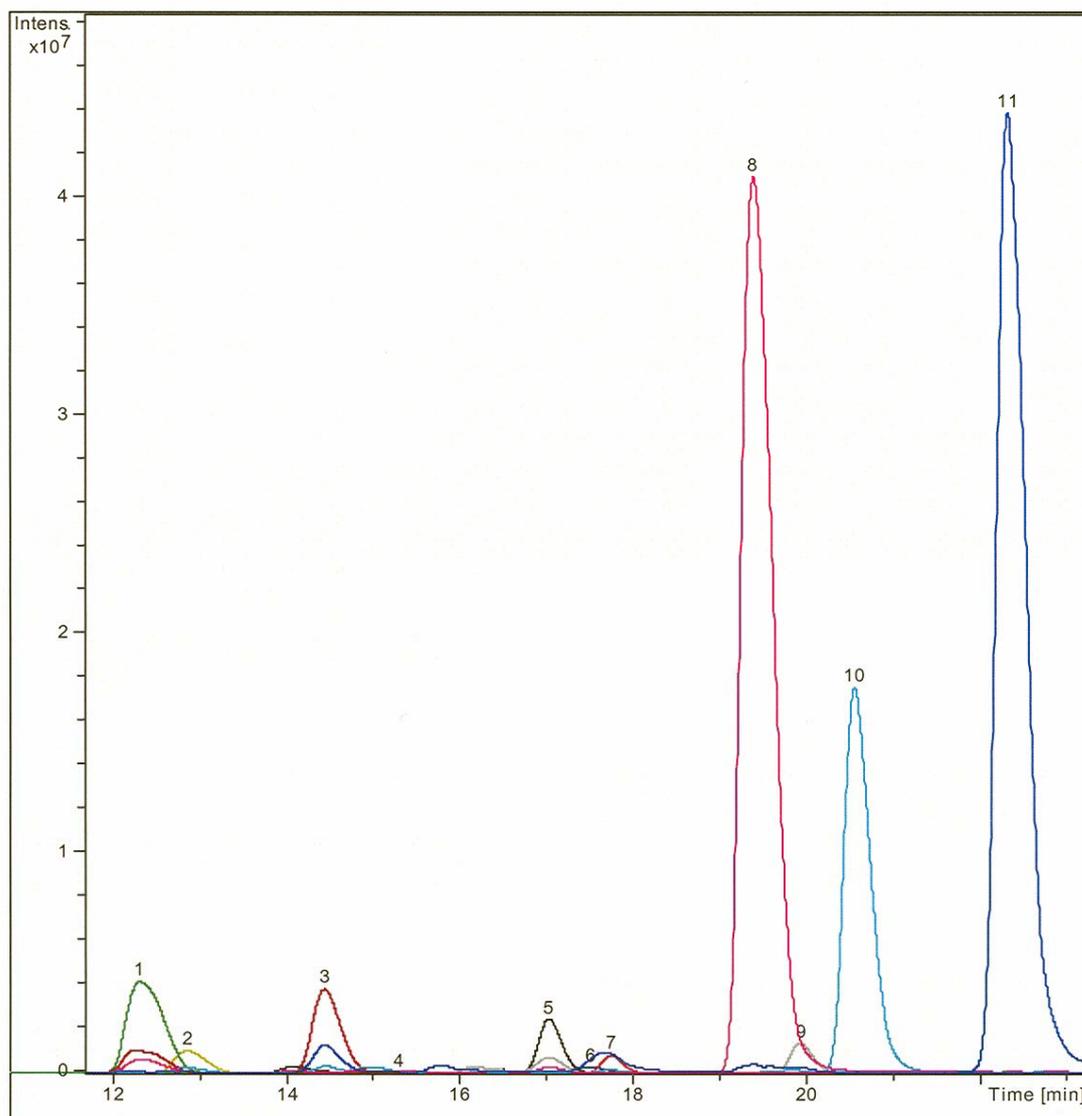


Figure 8.3 Electro-spray ionisation chromatogram of isoflavones using LC-MS in soymilk made from SPI; SUPRO 590, fermented with *B. animalis* ssp. *lactis* Bb12 after 12h at 37°C.

Elution order and peak identification	
1.	Daidzin (EIC; 417)
2.	Glycitin (EIC; 447)
3.	Genistin (EIC; 433)
4.	Malonyldaidzin (EIC; 503)
5.	Acetyldaidzin (EIC; 459)
6.	Malonylgenistin (EIC; 519)
7.	Acetylglycitin (EIC; 489)
8.	Daidzein (EIC; 255)
9.	Acetylgenistin (EIC; 475)
10.	Glycitein (EIC; 285)
11.	Genistein (EIC; 271)

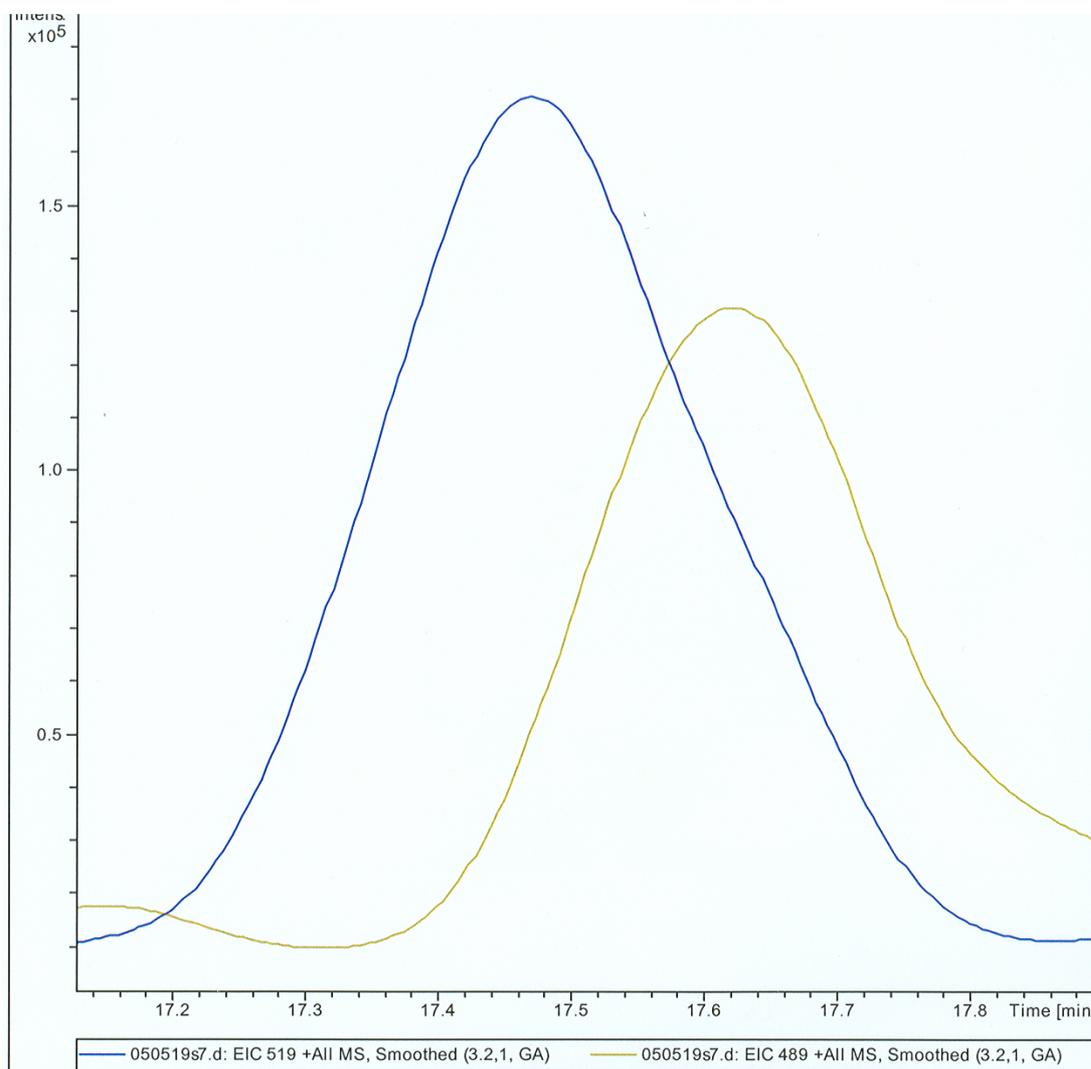


Figure 8.4 Separation and quantitation of malonylgenistin (bigger peak-EIC 519) and acetylglycitin (smaller peak-EIC 489) in fermented soymilk using LC-MS

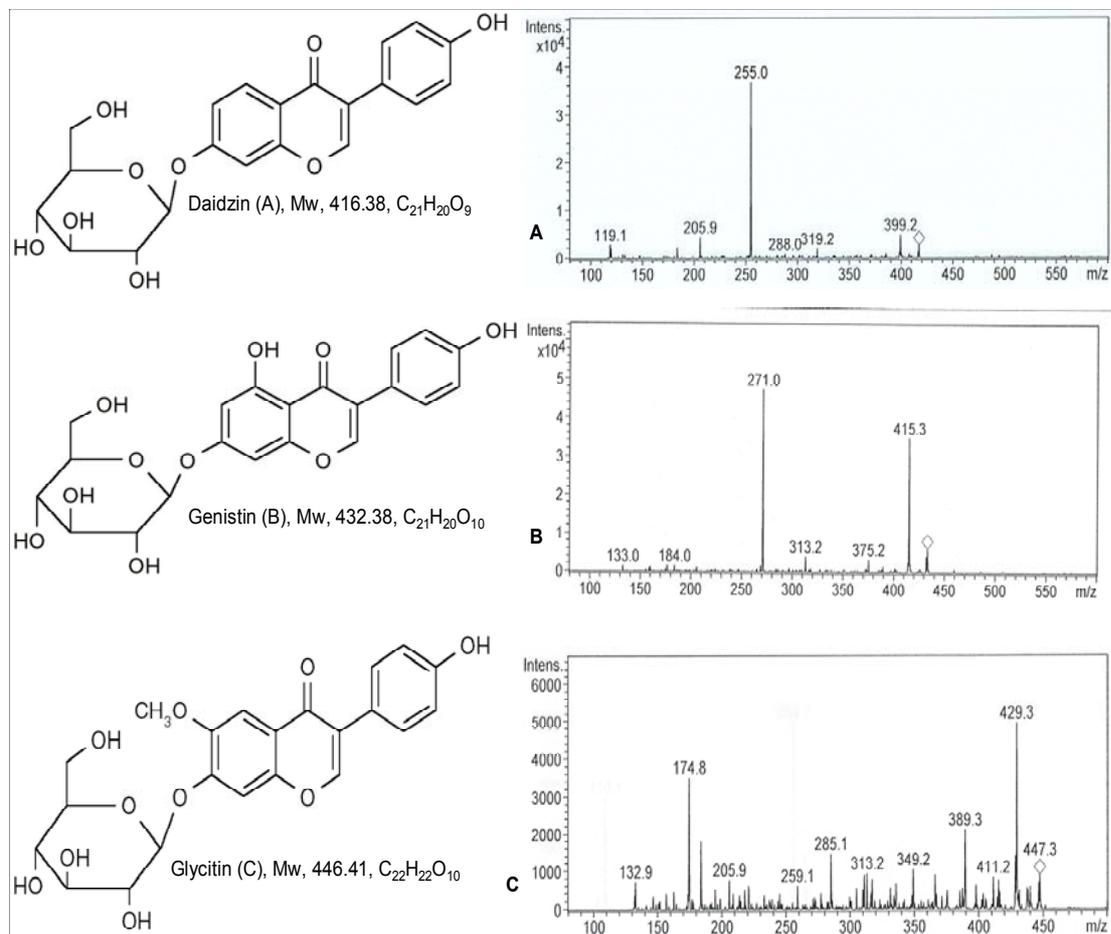


Figure 8.5 Products ions obtained in ESI-MS/MS experiments of protonated isoflavone glycosides ($M + H$) daidzin, genistin and glycitin (A, B, and C), respectively in soymilk. Parent ions indicated by the diamond mark.

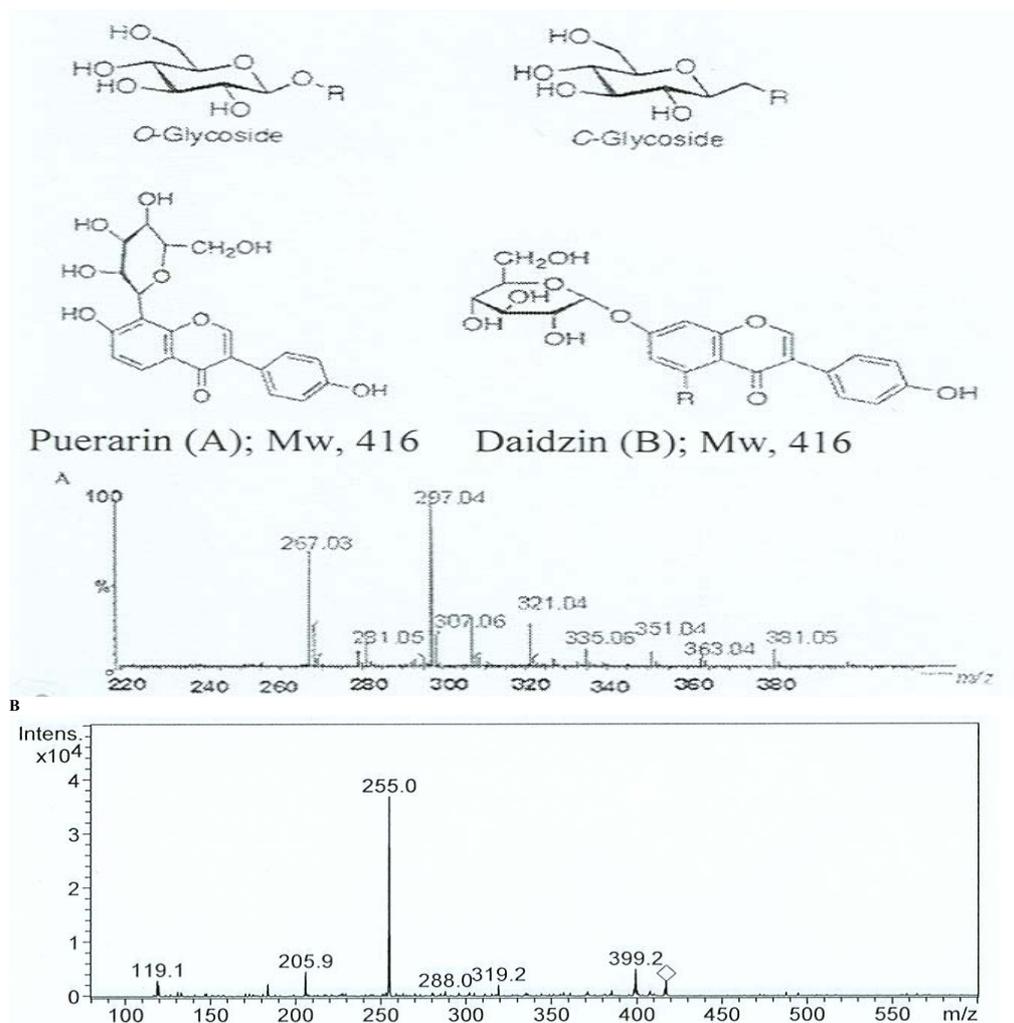


Figure 8.6 Comparison of the products ions obtained in ESI-MS/MS experiments of protonated standards of puerarin (A; *C*-glycosylated) and daidzin (B; *O*-glycosylated), respectively.

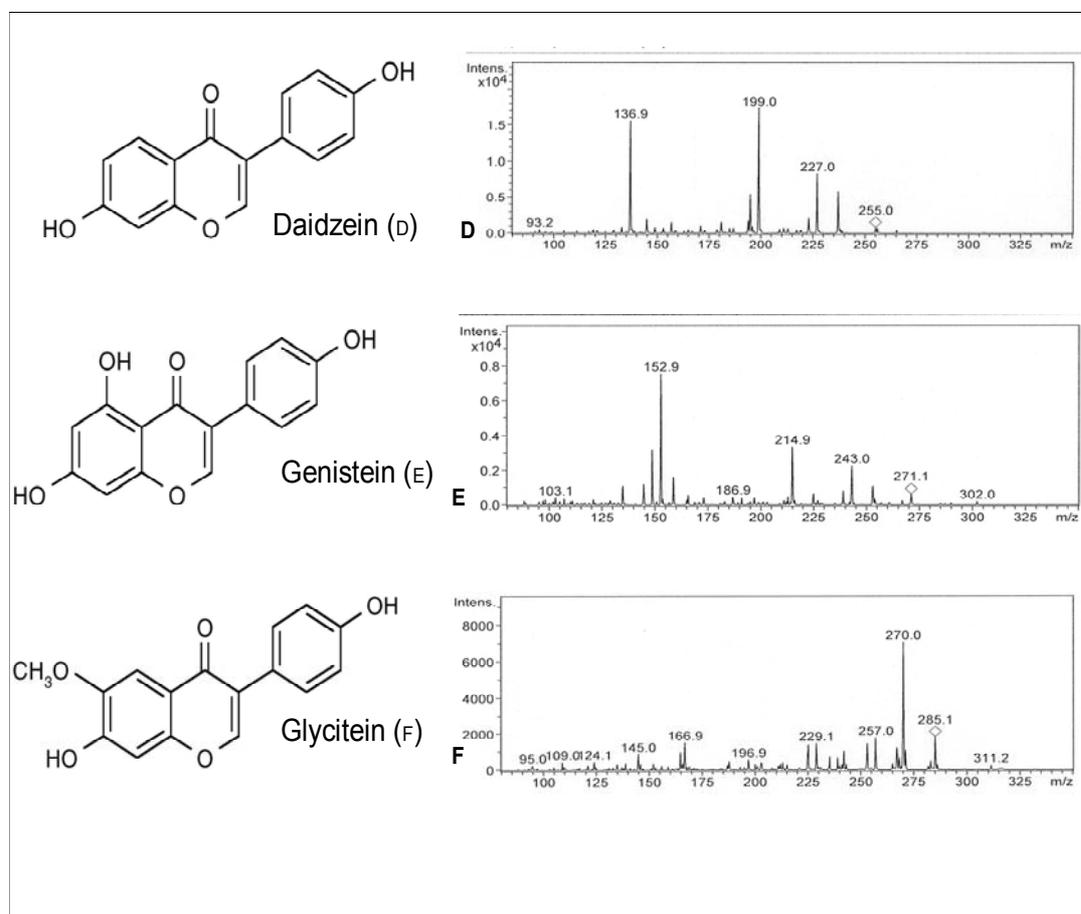


Figure 8.7 Products ions obtained in ESI-MS/MS experiments of protonated isoflavone aglycones daidzein, genistein and glycitein (D, E, and F) in soymilk, respectively. Parent ions are indicated by the diamond mark.

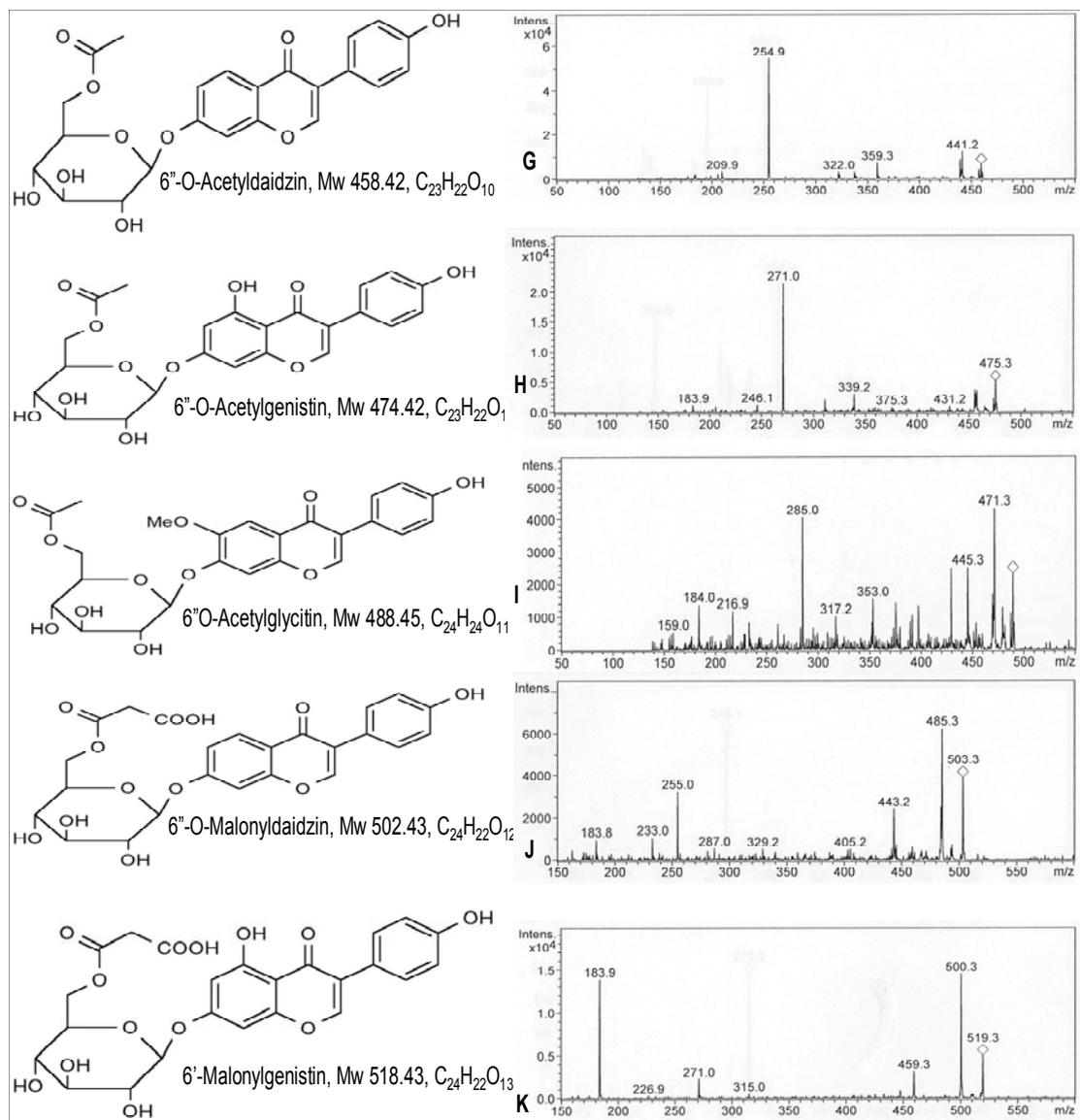


Figure 8.8 Products ions obtained in ESI-MS/MS experiments of protonated isoflavones of malonyl-, and acetyl glycosides (G, H, I, J, and K) product ions, respectively in soymilk. Parent ions are indicated by the diamond mark.

9.0 Endogenous β -Glucosidase and β -Galactosidase Activities from Selected Probiotic Microorganisms and their Role in Isoflavone Biotransformation in Soymilk

9.1 INTRODUCTION

Lactobacillus acidophilus, *Lactobacillus casei* and *Bifidobacterium* are important members of the normal intestinal flora in man and are recognized to be associated with the host's health (Tochikura *et al.* 1986). *Bifidobacterium* in conjunction with *L. acidophilus* are widely used in dairy preparations for their health benefits (Shah, 2000). In addition, they possess various forms of crude endogenous enzymes, some of which are important in the breakdown isoflavone glycosides. More importantly, these micro-organisms have been found to possess endogenous β -glucosidases (Chapter 3.0) which play a role in hydrolysis of isoflavone glycosides. Evaluation of the hydrolytic strength of probiotic micro-organisms based on β -glucosidase activity on isoflavone glycosides has recently been conducted (Chapter 4.0). Isoflavone conjugates after ingestion can be metabolised by the intestinal bacteria. However, due to variations in the level of intestinal bacteria through illnesses, diet or age (Hutt *et al.* 2006), the intestinal bacteria cannot always be relied upon for glycoside de-conjugation in order to release isoflavone aglycones. Accordingly, not every consumer could possibly derive the health benefits resulting from the formation of these bioactive compounds. Epidemiological studies comparing Asian and Caucasian diets and disease rates and dietary trials using soybean products, isoflavone or isoflavone concentrates have established with a reasonable degree of certainty that soy isoflavones have several useful biological activities in humans (Setchell, 2000).

The biological activity and metabolic fate of dietary soy isoflavones differ depending on their chemical forms (Cassidy, 1996). Since the structure itself is a limiting factor for absorption from gastrointestinal tract (Hendrich *et al.* 1999), the chemical forms of the isoflavones and their

metabolites influence the extent of absorption, with aglycones more readily absorbed and more bioavailable than highly polar conjugated species (Setchell, 2000). Isoflavones exist primarily in soybeans and in most soy foods as a complex mixture of glycoside conjugates that are not bioavailable in this form (Setchell *et al.* 2002a). In addition to the isoflavones, predominantly existing in glycoside forms, mature soybeans contain trace amounts of monosaccharides, such as glucose and arabinose, and measurable amounts of di- and oligosaccharides, with sucrose in the range of 2.5 – 8.2%; raffinose, 0.1 – 0.9%; and stachyose, 1.4 – 4.1% (Hymowitz *et al.* 1972). Basically, the oligosaccharides in soybeans are non reducing sugars, containing fructose, glucose, and galactose as two or more units, linked by β -fructosidic and α -galactosidic linkages. Therefore, isoflavones are glycosylated with a variety of sugars including glucose and galactose (Hymowitz *et al.* 1972).

The knowledge of the aglycone forms showing superior estrogenic effects over their respective glycosides *in vivo* has stimulated the development of aglycone-enriched products directly by β -glucosidase treatment (Park *et al.* 2002) or by fermentation with *Bifidobacterium* (Tsangalis *et al.* 2004). A study of Japanese women reported that isoflavone aglycones (a fermented soybean extract) were absorbed more efficiently than isoflavone glycosides (an unfermented soybean extract) (Izumi *et al.* 2000). It has also been reported that isoflavones from fermented products such as tempe, natto and soy sauce are more available to humans than from unfermented products (Hutchins *et al.* 1995; Slavin *et al.* 1998). In contrast, most recent studies reported that the bioavailability of daidzein and genistein isoflavones was greater when ingested as glycosides rather than as aglycones (Setchell *et al.* 2001). Two other studies found no significant differences in the absorption efficiency of aglycones and glycosides (Richelle *et al.* 2002; Zubik & Meydani, 2003). The aims of this study were to (a) examine the activity of crude endogenous β -glucosidase and β -galactosidase in selected microorganisms in fermented soymilk at 37°C over 24 h (b) assess the synergistic effects of the two enzymes on isoflavone biotransformation, and (c) determine the total aglycone yield per unit of total glycosides hydrolysed by the two endogenous enzymes.

9.2 MATERIALS AND METHODS

9.2.1 Bacterial strains

Pure cultures of *L. acidophilus* ATCC 4461 and *L. casei* 2607 and were obtained from the Victoria University Culture Collection (Werribee, Vic, Australia) and that of *B. animalis* ssp. *lactis* Bb12 was obtained from Chr. Hansen (Bayswater, Vic, Australia). The purity of the culture was confirmed by the procedure outlined in section 3.2.1. The stock cultures were stored at –80°C in sterile reconstituted skim milk (12% w/v) and 40% glycerol.

9.2.2 Soymilk manufacture

Soy protein isolate (SPI; SUPRO 590) supplied by The Solae Co. (Chatswood, NSW, Australia) was used in the production of soymilk according to the method of Tsangalis *et al.* (2002) as outlined in section 3.2.2. After reconstitution, the soymilk was dispensed into four glass bottles at 350 mL each, then autoclaved at 121°C for 15 min. The pH was adjusted to 6.7 aseptically using 5 M sodium hydroxide.

9.2.3 Assay for β -glucosidase and β -galactosidase enzymes from *L. acidophilus* ATCC 4461, *L. casei* 2607 and *B. animalis* ssp. *lactis* Bb12 and in soymilk

The 3 organisms were assessed for β -glucosidase and β -galactosidase activities in soymilk according to the method of Scalabrini *et al.* (1998) and in Chapter 3.0 (section 3.2.4) with some modifications. The microorganisms were activated in rehydrated de Mann Rogosa Sharpe (MRS) broth at 37°C for 20 h successively 3 times followed by a fourth activation in sterile soymilk using an inoculum level of 5% (v/v). Four hundred and forty millilitres of sterile soymilk were inoculated (in duplicate) with the active culture (5% v/v), and incubated at 37°C for 24 h to hydrolyse isoflavone glycosides in soymilk. Twenty millilitre aliquots of each sample were withdrawn aseptically at 0, 0.5, 1, 3, 6, 9, 12, 15, 18, 21 and 24 h for β -glucosidase and β -galactosidase assays while the remaining twenty millilitres were stored immediately at –80°C for

analysis of isoflavones. The frozen aliquots were freeze-dried using a Dynavac® FD300 freeze drier (Rowville, Vic, Australia) for isoflavone extraction and analysed using reverse-phase high-performance liquid chromatography (HPLC).

9.2.4 Quantification of β -glucosidase activity in soymilk

The enzyme was determined in soymilk by measuring the rate of hydrolysis of p -nitrophenyl β -D-glucopyranoside (p NPG). Of the 20 mL aliquot, 10 mL was used for the determination of β -glucosidase activity according to our method in section 3.2.4. Briefly, one thousand microlitres of 5 mM p NPG prepared in 100 mM sodium phosphate buffer (pH 7.0) was added to 10 mL of aliquot and incubated at 37°C for 15 min (Scalabrini *et al.* 1998). Five hundred microliters of 1M cold sodium carbonate were added to stop the reaction. The aliquots were then placed in 1.8 mL Eppendorf centrifuge tubes followed by centrifugation ($14,000 \times g$ for 30 min) using an Eppendorf centrifuge (model 5415C; Crown Scientific Pty, Ltd, Victoria, Australia). The amount of p -nitrophenol released was measured using a spectrophotometer (Pharmacia LKB®, Novospec II®, Uppsala, Sweden) at 420 nm. One unit of the enzyme activity was defined as the amount of β -glucosidase that released 1 micromole of p -nitrophenol from the substrate p NPG per millilitre per min under assay conditions.

9.2.5 Quantification of β -galactosidase activity in soymilk

The determination of β -galactosidase activity was conducted according to the method by Hsu *et al.* (2005) with some modification. Briefly, ten millilitres of aliquot were aseptically drawn at 0, 0.5, 1, 3, 6, 9, 12, 15, 18, 21 and 24 h of fermentation of soymilk with each microorganism. One thousand microliters of 15 mM O -nitrophenyl β -D-galactopyranoside ($ONPG$) in 0.03 M sodium phosphate buffer (pH 6.8) was added to the aliquot and incubated at 37°C for 15 min. The reaction was stopped by adding five hundred microliters of cold 0.1 M sodium carbonate. Absorbance was measured at 420 nm with a spectrophotometer (LKB NOVASPEC II,

Pharmacia, LKB Biochrom, England). A unit of β -galactosidase was defined as the amount of enzyme that catalyses the formation of 1 μ mol of o-nitrophenol per min under the assay condition.

9.2.6 Extraction of isoflavones from freeze dried samples

The extraction of isoflavones using HPLC grade methanol from freeze-dried sample and sample concentration for the HPLC runs was conducted following the procedure outlined in section 4.2.6.

9.2.7 HPLC analysis of isoflavones

HPLC isocratic elution was used to isolate the isoflavones for detection and the mobile phase consisted of 100% methanol and 10 mM ammonium acetate buffer (50:50) containing 1 mL of trifluoro-acetic acid per litre of solvent mixture. This was set at a flow rate of 0.95 mL/min according to our method in Chapter 5.0 (section 5.2.8). Injection volumes of isoflavone standards and of the samples were set at 20 μ L throughout the run time of 30 min. Alltech Alltima (Deerfield, IL, USA) HP C18HL (250 mm \times 4.6 mm internal diameter, 5 μ m) reversed-phase C18 column attached to an Alltima HP C18HL (7.5 mm \times 4.6 mm internal diameter, 5 μ m) guard column was used to separate the isoflavone compounds according to the our method in Chapter 5.0 (section 5.2.8). A single channel cell lead amperometric 641 VA detector was set at 800 mv and 0.5 μ A to detect malonyl-, acetyl-, and β -glycosides, and aglycones including equol, and flavone (ISTD). The analytes which can be oxidised or reduced at the potential reacts at the surface of the electrode as they pass through the flow cell. The detector monitors the addition or removal of electrons as an electric current. Single standards were prepared for peak identification. Malonyl- and acetyl-glycoside conjugates were quantified with respect to their β -glycoside equivalent response factors and corrected according to the molecular weight. Isoflavone concentrations were calculated back to wet basis (μ g/mL of soymilk).

9.2.8 Otieno – Shah (O – S) index

This was measured according to the method mentioned in Chapter 4.0 (Table 4.7) and refers to the ratio of β -glycoside reduction quotient to that of aglycone increment quotient. It is a measure of the hydrolytic potential of the enzyme in converting isoflavone glycosides to isoflavone aglycones in soymilk during fermentation. As fermentation proceeds, the concentration of isoflavone glycosides decreases while that of aglycones increases. At any given time (t) during fermentation, the glycoside concentration will always be less than its initial concentration (t = 0). Hence the glycoside reduction quotient (GRQ) at time (t) during fermentation can be determined by;

$$^a\text{Glycoside reduction quotient (GRQ)} = \frac{C_o}{C_t}$$

Where C_o is the initial glycoside concentration and C_t is the glycoside concentration at time t. For this reason, the GRQ will always be greater than 1. On the other hand, aglycone increment quotient (AIQ) refers to the ratio of isoflavone aglycone concentration at time (t) to that of its initial concentration at time (t = 0). It is determined by;

$$^b\text{Aglycone increment quotient (AIQ)} = \frac{C_t}{C_o}$$

Where C_t is the concentration at time (t) during fermentation or incubation while C_o is the initial concentration at time (t = 0). Since aglycone concentration increases with time due to hydrolysis, AIQ will also always be greater than 1. Otieno – Shah (O – S) index is therefore the ratio of GRQ to AIQ and is expressed as;

$$^c\text{Otieno – Shah (O-S) index } [c] = \frac{GRQ}{AIQ} \Leftrightarrow \left[\frac{a}{b} \right]$$

9.2.9 Statistical analysis of data

The quantification of isoflavones was carried out and the pH measurements were performed in triplicate on 3 trials. The data are means of 9 measurements and are presented as a mean \pm

standard error of 9 analyses. To find the difference in isoflavone concentrations in soymilk during storage at different temperatures, means were analysed using one-way analysis of variance (ANOVA) and 99% confidence levels using Microsoft[®] Excel Statpro[®] as described by Albright *et al.* (1999). ANOVA data with a $P < 0.01$ were classified as statistically significant.

9.3 RESULTS

9.3.1 β -Glucosidase activity of the probiotic microorganisms in soymilk

The activity of β -glucosidase from the selected probiotics in the soymilk at 37°C for 24 h is shown in Table 9.1. Highest β -glucosidase activity was obtained after 15 h of fermentation at 15.80 UmL⁻¹, 16.03 UmL⁻¹ and 16.05 UmL⁻¹ for *L. acidophilus* ATCC 4461, *L. casei* 2607 and *B. animalis* ssp. *lactis* Bb12, respectively.

9.3.2 β -Galactosidase activity of probiotic microorganisms in fermented soymilk

Table 9.2 shows β -galactosidase activity of the 3 microorganism in the soymilk during 24 h of fermentation. The highest enzyme activity at 0.97 UmL⁻¹, 0.97 UmL⁻¹ and 0.98 UmL⁻¹ was obtained for *L. acidophilus* ATCC 4461, *L. casei* 2607 and *B. animalis* ssp. *lactis* Bb12, respectively after 15 h of fermentation.

9.3.3 Isoflavone aglycone yields from glycoside hydrolysis

Increments in total isoflavone aglycone concentrations in the soymilk due to the hydrolytic activities of the two enzymes from each microorganisms are shown in Table 9.3. Although there was a synergistic hydrolysis of the isoflavone glycosides by the two enzymes, the vast difference in their activities during fermentation inferred that β -glucosidase had a greater role than β -galactosidase in glycoside hydrolysis.

9.3.4 Comparative increase of isoflavone aglycones from isoflavone glycosides in soymilk during hydrolysis by microbial enzymes

Figure 9.1 shows the total aglycone increment vis a vis total glycoside decrement values during fermentation with each microorganisms in the soymilk. There was a high aglycone yield from hydrolysis of a unit of isoflavone glycosides with all the three microorganisms.

9.3.5 Evaluation of the hydrolytic potential of the endogenous enzymes in isoflavone transformation in soymilk during 24 h fermentation

Figure 9.2 show the hydrolytic potential of each of the microorganisms in soymilk during fermentation of soymilk at 37°C for and 24 h and as Otieno Shah (O – S) index. Higher O – S index implies higher hydrolysing potential and vice versa. It is a precise way of evaluating the ability of microorganisms to hydrolyse isoflavone glycosides based on the activity of crude hydrolysing endogenous enzymes.

9.4 DISCUSSION

The data from our study showed that after 12 h of fermentation, β -glucosidase activity increased to almost 3 times the initial activity at inoculation for each of the microorganisms. As shown, there was a significant increase ($P < 0.01$) in the enzyme activity after 6 h of fermentation for each of the microorganism. There was, however, a further significant increase ($P < 0.01$) in the enzyme activity after 9 h, culminating at peak activity after 15 h for each of the 3 microorganims. This trend appeared to correlate with the growth cycle of each microorganims in the soymilk but was exemplified by the hydrolytic potential during fermentation as shown in Figure 9.2. Growth of probiotic cultures is generally slow compared with that of yoghurt starter cultures that take only 4 h to complete fermentation (Shah, 2000). It has been shown that certain sugars are preferentially assimilated by *Bifidobacterium* and *Lactobacillus* microorganisms for their growth and cellular metabolism. Probiotic microorganisms have been reported to hydrolyse varying p -nitrophenyl

glycosides and *Bifidobacterium* are known to possess higher β -glucosidase activity than most of the other microorganisms (Tochikura *et al.* 1986). Hence it was not entirely surprising that *B. animalis* ssp. *lactis* Bb12 exhibited higher β -glucosidase activity than *L. casei* 2607 and *L. acidophilus* ATCC 4461.

On the other hand, β -galactosidase activity was much lower than β -glucosidase in the soymilk for each of the organism. Comparatively, at peak activities occurring after 15 h for each microorganisms, the β -glucosidase activity was at 16.29, 16.52 and 16.38 times higher than that of β -galactosidase activity for *L. acidophilus* ATCC 4461, *L. casei* 2607 and *B. animalis* ssp. *lactis* Bb12, respectively. The trend of β -galactosidase activity, just like that of β -glucosidase activity also mirrored that of the growth cycle and potential to hydrolyse isoflavone glycoses during fermentation (Figure 9.2). The two endogenous enzymes were responsible for the hydrolysis of the β -1-6 glycoside bonds in the conjugated isoflavone glycoside forms (Chapter 4.0), predominant in unfermented soymilk. Determination of the activities of the two enzymes is therefore important as an indicator of the hydrolysing potential of the microorganism in releasing the bioactive isoflavone aglycone forms.

Most of the enzymes particularly α -galactosidase, β -galactosidase and β -glucosidase exist in crude forms in the probiotic microorganisms and therefore show activities towards ρ -nitrophenyl- α -D-galactopyranoside, o -nitrophenyl- β -D-glucopyranoside and ρ -nitrophenyl- β -D-glucopyranoside and amongst others (Tochikura *et al.* 1986). It is not clear at this point in time why β -glucosidase activity would be more than 15 times higher than that of β -galactosidase in soymilk as they hydrolyse similar bonds. One possible reason for the lower β -galactosidase activity could be related to the concentration of β -D-galactopyranosides (such as lactose) found in the soymilk. Since there is no lactose in soymilk, this could be a direct limiting factor in exhibiting β -galactosidase activity and there is no substrate challenge limiting production of the enzyme. The amount of a certain specific substrate present in the soymilk could evoke a corresponding specific enzyme activity. It is possible that the occurrence of a relatively higher

amount of glucose compared to lactose could stimulate a higher β -glucosidase activity than β -galactosidase activity. Since there is a certain occurrence of β -D-glucopyranoside substrate as other glycoside in soymilk, it could stimulate higher β -glucosidase activity than β -galactosidase activity. Among the soluble carbohydrates found in soybeans (e.g. glucose and sucrose), raffinose and stachyose usually receive more attention because their presence is linked with the flatulence and abdominal discomfort associated with soybean and soy food consumption (Liu, 1997). It is also reasonable to infer that isoflavones in the soymilk could be glycosylated with a variety of sugars including glucose and galactose.

Several investigators have described the carbon source regulation of β -galactosidase biosynthesis in various microorganisms (De Vries *et al.* 1999; Nagy *et al.* 2001; Fekete *et al.* 2002). They indicated that the role of carbon source in the biosynthesis of β -galactosidase may vary and depends on the microorganism tested. Hsu *et al.* (2005) demonstrated that lactose is the best carbon source for inducing maximum β -galactosidase activity, while glucose is a poor inducer. Similar observations were made by Fiedurek and Szczodrak (1994) for *Kluyveromyces fragilis* and Shaikh *et al.* (1997) for *Rhizomucor* sp. Thus, amounts of carbon source in the medium may actually affect the expression of β -galactosidase by the microorganism (Fiedurek & Szczodrak, 1994; Inchaurredo *et al.* 1998). Therefore, in order to stimulate higher β -galactosidase activity from probiotic microorganisms and enhance its role in the isoflavone hydrolysis, addition of lactose to the soymilk may be necessary. Lactose intolerance among dairy products by a huge population of consumers is a major reason for the increasing consumption of soymilk and its beverages. According to Hsu *et al.* (2005), the other determinant of β -galactosidase activity from microorganisms is the nitrogen source. Yeast extracts were found to stimulate highest production of β -galactosidase activity by *Bifidobacterium longum* CCRC 15708 compared to other nitrogen sources such as casein, peptone, tryptone, gelatine and beef extract. Thus without stimulants for β -galactosidase activity such as nitrogen and carbon sources in soymilk, it is clear that the role of

β -galactosidase activity in the hydrolysis of isoflavone glycosides was far too limited compared to that of β -glucosidase enzyme.

For each microorganism, there was also a significant increase ($P < 0.01$) of isoflavone aglycones just after 0.5 h during incubation at 37°C. However, the most significant increase ($P < 0.01$) occurred at 15 h with each microorganism. There was a small but insignificant reduction ($P < 0.01$) after reaching peak concentration at 15 h, just as the pattern of the activities of β -glucosidase and β -galactosidase enzymes. Hence there was a direct correlation between aglycone increment and the activities of the two enzymes in the soymilk during fermentation with each of the three microorganisms. From this relationship, it is clear that β -glucosidase, having a much higher activity than β -galactosidase had a proportionately greater role in the hydrolysis of isoflavone glycosides, which are predominant in unfermented soymilk. It appears that at least 9 h of fermentation was required for significantly improving the biological activity of soymilk, but 15 h would be the optimum for these microorganisms. As shown the optimum fermentation time was further highlighted by the hydrolytic potential of each microorganism on isoflavone glycosides expressed as Otieno Shah (O – S) index.

The hydrolytic potential of each microorganism varied with fermentation time but was dependent on the activities of the hydrolysing enzymes (in this case β -glucosidase and β -galactosidase). Maximum hydrolysis occurred at 15 h for each microorganism and is therefore the best indicator of the length of fermentation needed to be carried out. There was little difference between the 3 microorganisms, although *B. animalis* ssp. *lactis* Bb12 had a slightly higher hydrolytic potential than *L. casei* 2607 and *L. acidophilus* ATCC 4461 at 15 h of fermentation (Figure 9.2).

During hydrolysis, the concentration of glycosides such as genistin, glycitin and daidzin together with the corresponding malonyl, - and acetyl glycosides reduced while the concentration of aglycones such as genistein, glycitein and daidzein increased. The hydrolysis of a unit of total glycosides comparatively yielded more units of total aglycones. For example, at peak total aglycone concentration, the total glycosides reduced by a factor of 0.79 while yielding an

increase in the total aglycones by a factor of 6.10 in the soymilk fermented by *B. animalis* ssp. *lactis* Bb12. The conversion of individual forms of isoflavone glycosides to respective aglycones has been reported in our previous work (Chapters 4.0 to 8.0). While there were variations in concentration of aglycone from respective isoflavone glycosides, glycitin to glycitein conversion was found to be lowest as reported earlier (Chapters 4.0 to 8.0 and 10.0), possibly due to steric hindrance.

9.5 CONCLUSIONS

B. animalis ssp. *lactis* Bb12 had a slightly higher β -glucosidase activity, β -galactosidase activity, aglycone increment value and therefore a higher hydrolytic potential than *L. acidophilus* ATCC 4461 and *L. casei* 2607 at 15 h of soymilk fermentation. The three microorganisms also showed a similar trend of increase in β -glucosidase activity, β -galactosidase activity and aglycone increment value during fermentation. A fermentation time of 15 h is recommended in order to improve the biological activity of soymilk for these probiotic microorganisms.

Table 9.1 β -Glucosidase activity (UmL⁻¹) of selected probiotics in fermented soymilk at 37°C for 24 h

Time (h)	<i>L. acidophilus</i> 4461	<i>L. casei</i> 2607	<i>B. animalis</i> Bb12
0	0	0	0
0.5	6.72 ± 1.2 ^{BC}	5.65 ± 1.5 ^{BC}	6.39 ± 1.2 ^{BC}
1	9.67 ± 3.3 ^{BC}	9.81 ± 3.3 ^{BC}	9.88 ± 3.4 ^{BC}
3	10.29 ± 3.6 ^{BC}	10.18 ± 3.6 ^{BC}	10.17 ± 3.6 ^{BC}
6	12.74 ± 6.1 ^{BCD}	13.35 ± 5.7 ^{BCD}	13.42 ± 5.7 ^{BCD}
9	14.31 ± 4.4 ^{BCDF}	14.22 ± 4.5 ^{BCDF}	14.55 ± 4.3 ^{BCDF}
12	15.60 ± 6.2 ^{BCDE}	14.70 ± 5.1 ^{BCDEF}	15.04 ± 5.4 ^{BCDEF}
15	15.80 ± 5.9 ^{BCDEF}	16.03 ± 5.9 ^{BCDE}	16.05 ± 5.9 ^{BCDE}
18	14.74 ± 5.3 ^{BCDEF}	14.70 ± 6.2 ^{BCDEF}	14.73 ± 6.3 ^{BCDEF}
21	14.35 ± 5.3 ^{BCDF}	14.35 ± 5.3 ^{BCDF}	14.17 ± 5.3 ^{BCDF}
24	13.19 ± 6.2 ^{BCDF}	13.24 ± 6.1 ^{BCDF}	13.14 ± 6.2 ^{BCDF}

^{ABCDEF} Means in the same column for the strains with different capital letter superscripts are significantly different

One unit of enzyme activity was defined as the amount of enzyme required to release 1 μ mol of p-nitrophenol from p-nitrophenyl- β -D-glucopyranoside per millilitre per min under assay conditions. Results are presented as a mean of 6 observations. Level of significance was set at $P < 0.01$. P values for *L. casei* 2607 = 0.4348, *L. acidophilus* 4461 = 0.4576, *B. animalis* ssp. *lactis* Bb12 = 0.3432

Table 9.2 β -Galactosidase activity (UmL⁻¹) of selected probiotics in fermented soymilk at 37°C for 24 h

Time (h)	<i>L. acidophilus</i> ATCC 4461	<i>L. casei</i> 2607	<i>B. animalis ssp. lactis</i> Bb12
0	0	0	0
0.5	0.52 ± 0.27 ^B	0.51 ± 0.28 ^B	0.63 ± 0.36 ^B
1	0.60 ± 0.2 ^B	0.60 ± 0.22 ^B	0.70 ± 0.28 ^{BC}
3	0.91 ± 0.19 ^{BCD}	0.91 ± 0.18 ^{BCD}	0.95 ± 0.21 ^{BCD}
6	0.93 ± 0.29 ^{BC}	0.86 ± 0.20 ^{BC}	0.88 ± 0.22 ^{BC}
9	0.90 ± 0.28 ^{BC}	0.92 ± 0.27 ^{BC}	0.83 ± 0.23 ^{BC}
12	0.89 ± 0.23 ^{BC}	0.94 ± 0.28 ^{BC}	0.82 ± 0.23 ^{BC}
15	0.97 ± 0.40 ^{BCD}	0.97 ± 0.27 ^{BCD}	0.98 ± 0.31 ^{BCD}
18	0.90 ± 0.30 ^{BC}	0.93 ± 0.33 ^{BC}	0.90 ± 0.31 ^{BCD}
21	0.80 ± 0.23 ^{BCD}	0.92 ± 0.30 ^{BCD}	0.79 ± 0.18 ^{BCDF}
24	0.77 ± 0.23 ^B	0.87 ± 0.29 ^B	0.76 ± 0.21 ^B

^{ABCDEF} Means in the same column for the strains with different capital letter superscripts are significantly different

One unit of enzyme activity was defined as the amount of enzyme required to release 1 μ mol of o-nitrophenol from o-nitrophenyl- β -D-glucopyranoside per millilitre per min under assay conditions. Results are presented as a mean of 6 observations. Level of significance was set at P < 0.01. P values for *L. casei* 2607 = 0.2669, *L. acidophilus* 4461 = 0.3305, *B. animalis ssp. lactis* Bb12 = 0.3559

Table 9.3 Increments of total aglycone concentration in soymilk during 24 h incubation with selected probiotic micro-organisms

Fermentation time (h)	<i>L. acidophilus</i> ATCC 4461	<i>L. casei</i> 2607	<i>B. animalis</i> ssp. <i>lactis</i> Bb12
0	1 ^A	1 ^A	1 ^A
0.5	1.76 ^{AB}	1.65 ^{AB}	1.89 ^{AB}
1	2.25 ^B	1.98 ^B	2.20 ^B
3	3.21 ^B	3.19 ^B	3.70 ^B
6	4.35 ^B	5.34 ^B	6.01 ^B
9	5.34 ^{BC}	5.34 ^{BC}	6.02 ^{BC}
12	5.37 ^{BC}	5.48 ^{BC}	6.08 ^{BC}
15	5.37 ^{BC}	5.52 ^{BC}	6.10 ^{BC}
18	5.31 ^{BC}	5.35 ^{BC}	5.99 ^{BC}
21	5.31 ^{BC}	5.34 ^{BC}	5.89 ^{BC}
24	5.23 ^{BC}	5.19 ^{BC}	5.83 ^{BC}

Table values – Number of times increment of the initial aglycone concentration in soymilk

^{ABC}Means in the same column with different superscripts are significantly different.

^{ABC}Means across the rows with different superscripts are also considered significantly different.

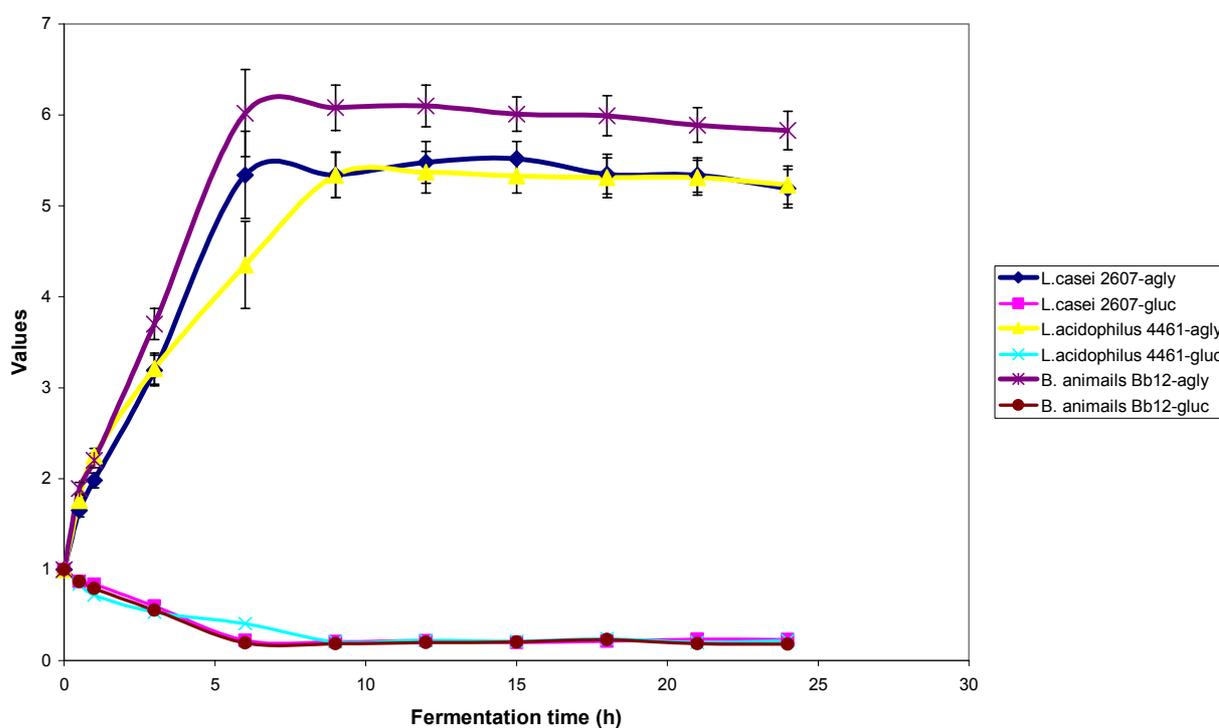


Figure 9.1 Total aglycone increment and total glycoside decrement values in fermented soymilk with selected probiotic microorganisms.

Results expressed as mean \pm standard deviation (SD) (n = 6).

Total aglycones = sum of genistein, glycitein and daidzein detected in soymilk during the fermentation process

Total glycosides = sum of genistin, glycitin and daidzin and respective acetyl;- and malonylglycosides detected in soymilk during the fermentation process.

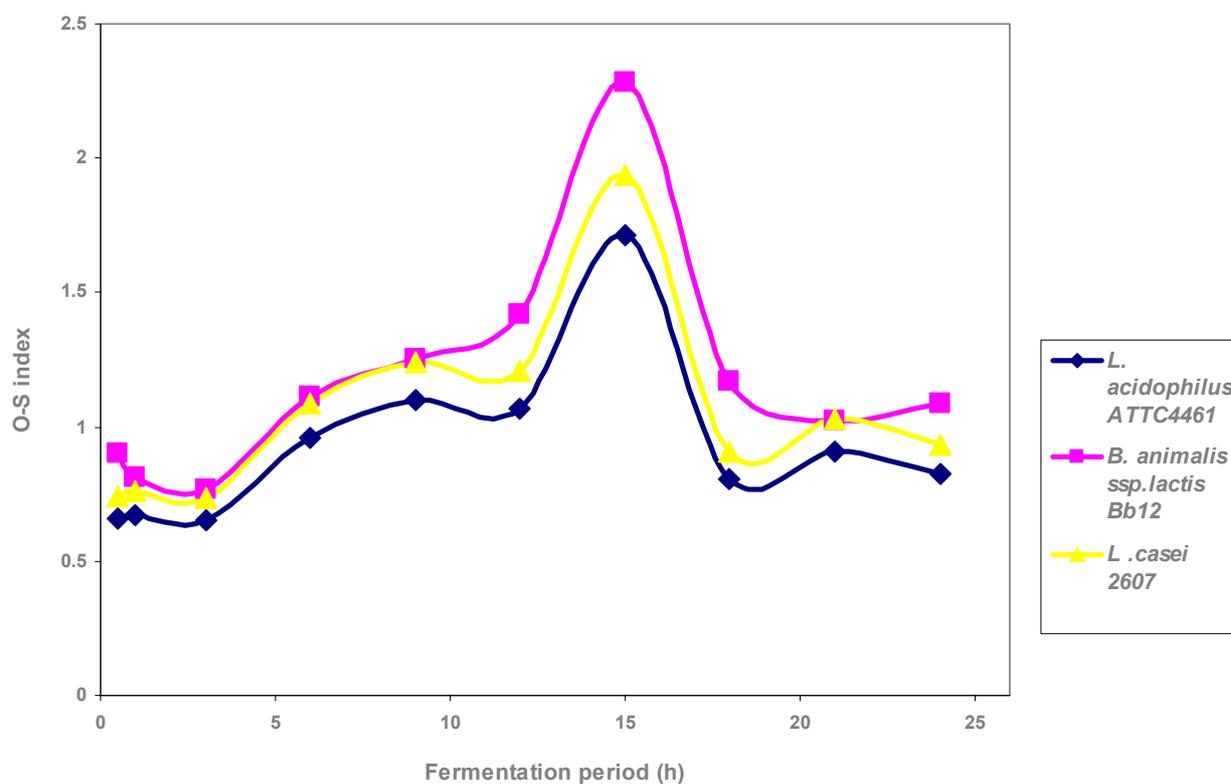


Figure 9.2 Hydrolytic indices (Otieno Shah (O-S) indices) of isoflavone hydrolysis in soymilk using probiotic micro-organisms during 24 h fermentation at 37°C.

Otieno-Shah (O-S) index (isoflavone hydrolytic index)- refers to the ratio of β -glycoside reduction quotient to the aglycone increment quotient.

10.0 A comparison of changes in transformation of isoflavones in soymilk using varying concentrations of exogenous and probiotic-derived endogenous β -glucosidases

10.1 INTRODUCTION

Although present in several legumes, soy beans are the principal human dietary source of isoflavones. Soy bean derived protein extracts and foods available for human consumption contain significant levels of isoflavones (Cassidy *et al.* 2000). There is a large variability and profile of isoflavones amongst soy products depending on species, geographical and environmental conditions, and the extent of industrial processing (Wang & Murphy 1994a; 1994b). Isoflavones occur in soymilk either as aglycones or as glycosides, including acetyl,-and malonylglycosides (Tsangalis *et al.* 2002). The relative proportions of glycoside conjugates can vary considerably between different soy foods. Isoflavones exist primarily in soybeans and in most soy foods as a complex mixture of glycoside conjugates that are not bioavailable in this form (Setchell *et al.* 2002a). Isoflavone conjugates after ingestion are either hydrolysed by the acid in the stomach or metabolised by the intestinal bacteria (Setchell 1998). However, due to variations in the level of intestinal bacteria through illnesses, diet or age (Hutt *et al.* 2006), the intestinal bacteria cannot always be relied upon for glycoside de-conjugation in order to release isoflavone aglycones. After ingestion, the isoflavone glycosides are hydrolysed by both intestinal mucosal- and bacterial β -glucosidases releasing the aglycones (Setchell *et al.* 2002a), which are then either absorbed directly or further metabolised by the intestinal microbiota in the large intestine into other metabolites, including equol (Setchell *et al.* 2002b) and O-desmethylangolensin (ODMA) (Joannou *et al.* 1995). Accordingly, not every consumer could possibly derive the health benefits from these bioactive compounds. Probiotic micro-organisms

----- Chapter 10.0 A comparison of exogenous and endogenous β -glucosidases including *Bifidobacterium* are widely used in dairy preparations in conjunction with *L. acidophilus* for their health benefits (Shah 2000). Bifidobacteria are among the predominant members of the intestinal microbiota. Some of these organisms are being classified as probiotics which enable their widespread use in the dairy industry (Hutt *et al.* 2006). More importantly, these micro-organisms have been found to possess endogenous β -glucosidases (Chapter 3.0 & Chapter 9.0) and can play an important role in improving the biological activity of soymilk. Evaluation of the hydrolytic strength of probiotic micro-organisms based on β -glucosidase activity on isoflavone glycosides has recently been conducted (Chapter 4.0).

The knowledge of the aglycone forms showing superior estrogenic effects over their respective glycosides *in vivo* has stimulated the *in vitro* development of aglycone-enriched products directly by β -glucosidase treatment (Park *et al.* 2002, 2003) or by fermentation with *Bifidobacterium* (Tsangalis *et al.* 2002).

A study of Japanese women reported that isoflavone aglycones (a fermented soybean extract) were absorbed more efficiently than isoflavone glycosides (an unfermented soybean extract) (Izumi *et al.* 2000). It has also been reported that isoflavones from fermented products such as *tempe*, *natto* and soy sauce are more available to humans than from unfermented products (Hutchins *et al.* 1995). In contrast, most recent studies reported that the bioavailability of daidzein and genistein isoflavones was greater when ingested as glycosides rather than as aglycones (Setchell *et al.* 2001). Two other studies found no significant differences in the absorption efficiency of aglycones and glycosides (Richelle *et al.* 2002; Zubik & Meydani 2003). Epidemiological studies comparing Asian and Caucasian diets and disease rates and dietary trials using soybean products, isoflavone or isoflavone concentrates have established with a reasonable degree of certainty that soy isoflavones have several useful biological activities in humans (Setchell 2000). The majority of these studies over the years involved forms of soy or soy extracts containing typical natural ratios of genistein:daidzein:glycitein based components of

----- Chapter 10.0 A comparison of exogenous and endogenous β -glucosidases approximately 1.3:1.0:0.2 also expressed as the aglycone equivalent (www.lclabs.com 2005). The use of soy isoflavones containing different ratios of the aglycone equivalents in clinical studies could be the reason for conflicting conclusions on the bioavailability of isoflavones. A knowledge of the changing aglycone equivalents in soymilk during fermentation or incubation with exogenous enzymes could help provide further understanding on the bioavailability of different forms of isoflavone leading to maximum health benefits. Therefore, the objectives of this study were to compare the kinetics of isoflavone transformation in soymilk using exogenous and endogenous β -glucosidases and monitor the aglycone equivalent ratios and aglycone concentrations during the fermentation process.

10.2 MATERIALS AND METHODS

10.2.1 Bacterial strains

Pure cultures of *L. casei* 2607 and *L. acidophilus* ATCC4461 were obtained from the Victoria University Culture Collection (Werribee, Vic, Australia) and that of *B. animalis* ssp. *lactis* Bb12 was obtained from Chr. Hansen (Bayswater, Vic, Australia). The purity of the culture was confirmed by the procedure outlined in section 3.2.1. Storage of the cultures were at -80°C in sterile reconstituted skim milk (12% w/v) and 40% glycerol.

10.2.2 Soymilk manufacture

Soy protein isolate (SPI; SUPRO 590) supplied by The Solae Co. (Chatswood, NSW, Australia) was used in the production of soymilk according to the method of Tsangalis *et al.* (2002) as outlined in section 3.2.2. Sterilisation of the reconstituted, aseptic transfer into bottles and pH adjustments were conducted according to the process mentioned in section 9.2.2.

10.2.3 Preparation of enzyme solutions and assay in soymilk

Pure lyophilised β -glucosidase (E.C. 232-589-7) extracted from almond was purchased from Sigma Chemicals (Castle Hill, NSW, Australia). The enzyme was dissolved in ice cold 0.05 M tris-HCl (tris hydroxymethyl aminomethane hydrochloride) buffer, pH 7.8 (about 1 mg/mL) and made into various concentrations (0, 0.072, 0.144, 0.288, 0.359, and 0.575 U mL⁻¹) using 0.2% bovine serum albumin (E.C. 232-9362) (Sigma).

10.2.4 Fermentation of soymilk with *B. animalis* ssp. *lactis* Bb12, *L. casei* 2607 and *L. acidophilus* ATCC 4461 and with exogenous β -glucosidase

The micro-organisms were activated in rehydrated de Mann Rogosa Sharpe (MRS) broth at 37°C for 20 h successively 3 times followed by a fourth activation in sterile soymilk according to our previous method in Chapter 4.0, section (4.2.5). An inoculum level of 5% (v/v) and the incubation at 37°C for 20 h were used for activation. Three hundred and fifty millilitres of sterile soymilk were inoculated (in duplicate) with the active culture (5% v/v), and incubated at 37°C for 4 h to hydrolyse isoflavone glycosides in soymilk. Similarly, known concentrations of exogenous β -glucosidase solution were also added to a known volume of soymilk (0.2% v/v), and incubated in a water bath at 37°C for up to 4 h. Fifty millilitre aliquots of exogenous and endogenous samples were withdrawn aseptically at 0, 15 min, 30 min, 1 h, 2 h, 3 h, and 4 h and stored immediately at -80°C for analysis of isoflavones. The frozen aliquots were freeze-dried using a Dynavac® FD300 freeze drier (Rowville, Vic, Australia) for isoflavone extraction and analysed using reverse-phase high-performance liquid chromatography (HPLC).

10.2.5 Extraction of isoflavones from freeze dried samples

The extraction of isoflavones using HPLC grade methanol from freeze-dried sample, refluxing and filtration conditions were same as those outlined in section 4.2.6. After drying, a 5 mL

----- Chapter 10.0 A comparison of exogenous and endogenous β -glucosidases aliquot was taken and after adding 60 μ L of internal standard (ISTD) flavone solution (10 mg/50 mL), the sample was dried in an Edwards Dynavac speed vacuum dryer SC110 connected to a Savant refrigerated condensation trap RT400 (Sussex, England). The resultant dried matter was then resuspended in 1 mL of 10 mM ammonium acetate buffer (containing 0.1% trifluoro-acetic acid) and methanol (50:50) solution and centrifuged ($14,000 \times g$ for 30 min) using an Eppendorf centrifuge (model 5415C; Crown Scientific Pty. Ltd., Vic, Australia), prior to transferring to HPLC vials.

10.2.6 Isoflavone standards

All the isoflavone standards including genistein, daidzein and glycitein as well as flavone (ISTD) and the β -glycoside standards of genistin, daidzin and glycitin as well as daidzein metabolite equol were purchased from sources mentioned in section 4.2.7. Genistein, genistin, flavone, daidzein, and equol were prepared in HPLC grade methanol, and daidzin, glycitein and glycitin in ethanol due to their varied solubility characteristics.

10.2.7 HPLC analysis of isoflavones

HPLC isocratic elution was used to isolate the isoflavones for detection and the mobile phase consisted of 100% methanol and 10 mM ammonium acetate buffer (50:50) containing 1 mL of trifluoro-acetic acid per litre of solvent mixture. This was set at a flow rate of 0.95 mL/min according to our method in Chapter 5.0 (section 5.2.8). Injection volumes of isoflavone standards and of the samples, HPLC run time, column specifications and guard column and its specifications were according to our method outlined in chapter 5.0 (section 5.2.8). A single channel cell lead Amperometric 641 VA detector was set at 800 mv and 0.5 μ A to detect malonyl-, acetyl-, and β -glycosides, and aglycones including equol, and flavone (ISTD). The analytes which can be oxidised or reduced at the potential reacts at the surface of the electrode as

----- Chapter 10.0 A comparison of exogenous and endogenous β -glucosidases they pass through the flow cell. The detector monitors the addition or removal of electrons as an electric current. Single standards were prepared for peak identification. Isoflavone concentrations were calculated back to wet basis ($\mu\text{g}/\text{mL}$ of soymilk).

10.2.8 Otieno – Shah (O – S) index

This was measured according to the method mentioned in Chapter 4.0 (Table 4.7). Calculation of glycoside reduction quotient (GRQ), Aglycone increment quotient (AIQ) as well as Otieno – Shah (O-S) index followed the outline and definitions mentioned in Chapter 9.0 (Section 9.2.8).

10.2.9 Statistical analysis of data

The quantification of isoflavones was carried out and the pH measurements were performed in triplicate on 2 trials. The data are means of 6 measurements and are presented as a mean \pm standard error of 6 analyses. To find the difference in isoflavone concentrations in soymilk during storage at different temperatures, means were analysed using one-way analysis of variance (ANOVA) and 99% confidence levels using Microsoft[®] Excel Statpro[®] as described by Albright *et al.* (1999). ANOVA data with a $P < 0.01$ were classified as statistically significant.

10.3 RESULTS

10.3.1 Changes in isoflavone concentration in soymilk using exogenous β -glucosidase

Based on the hydrolysing ability of the exogenous enzymes on isoflavone glycosides, we selected three levels of concentration resulting into β -glucosidase activity of 0.288 UmL^{-1} , 0.359 UmL^{-1} and 0.575 UmL^{-1} . Incubation of soymilk was conducted at 37°C for up to 4 h in order to determine the trends in isoflavone transformation. Figure 10.1 shows the effects of β -glucosidase enzyme with activity of 0.288 UmL^{-1} on 50 mL of soymilk during 4 h of incubation.

----- Chapter 10.0 A comparison of exogenous and endogenous β -glucosidases

Figure 10.2 shows the changes in isoflavone concentration in soymilk during incubation using β -glucosidase solution with an activity of 0.359 UmL^{-1} while Figure 10.3 shows the changing concentrations of isoflavone compounds in soymilk with β -glucosidase solution having an activity of 0.575 UmL^{-1} . All isoflavone forms including malonyldaidzin, malonylgenistin, acetyldaidzin and acetylgenistin were detected and quantified except malonylglycitrn and acetylglycitin in the soymilk made from SPI SUPRO 590. The acetyl and malonylglycosides shown in Figures 10.1, 10.2 and 10.3 were the sum total of the individual acetyl and malonyl forms. Also, in Figures 10.1, 10.2 and 10.3, the aglycone-glycoside equilibrium point occurs after about 8 mins for exogenous β -glucosidase while the same occurs for endogenous enzymes (Figures 10.6, 10.7 and 10.8) after about 125 mins.

10.3.2 Aglycone equivalent ratios for isoflavone aglycones in soymilk

Table 10.1 shows the effect of different concentrations of exogenous enzymes and microbial endogenous enzymes on the aglycone equivalent ratios with incubation time. For example, SPI SUPRO 590 from which soymilk was manufactured had a pre-hydrolysis aglycone equivalent ratio of 0.89:1.0:0.11, for genistein:daidzein:glycitein, respectively. We show in Table 10.1 that the aglycone ratio in soymilk changes with time and the time it takes to attain optimal native-like aglycone ratio is influenced by enzyme activity of the hydrolysing β -glucosidase. Aglycone equivalent ratio is the ratio of isoflavone aglycone genistein:daidzein:glycitein and can vary with incubation or fermentation time.

10.3.3 Comparison of the effects of exogenous and endogenous β -glucosidase in increasing the aglycone concentration in the soymilk during the 4 h incubation

Table 10.2 shows the concentration of total isoflavone aglycones in soymilk during incubation with exogenous and endogenous β -glucosidases. The total concentration of glycosides including

----- Chapter 10.0 A comparison of exogenous and endogenous β -glucosidases the malonylglycosides of genistein, daidzein and glycitein; their acetylglycosides as well as their free glycosides was 85.9% while the total aglycone composition consisted of the remaining 14.1%.

10.3.4 Evaluation of the hydrolytic potential of exogenous and endogenous enzymes in isoflavone transformation in soymilk

Figures 10.4 and 10.5 show the evaluation of the hydrolytic potential of both exogenous and endogenous enzyme after 4 h of incubation and 24 h of fermentation of soymilk at 37°C and are expressed as O – S index. Higher O – S index implies higher hydrolysing potential and vice versa.

Figures 10.6, 10.7 and 10.8 show the concentration of isoflavones in the soymilk during 24 h fermentation with the three micro-organisms. Interestingly, after 15 h during which O – S indices was highest, the aglycone increment levels also appeared to be highest. The aglycone increment for *B. animalis* ssp. *lactis* Bb12, *L. acidophilus* ATCC4461 and *L. casei* 2607 were 5.75, 5.72 and 5.95 fold, respectively.

10.4 DISCUSSION

The data presented show that incubation of soymilk with added exogenous β -glucosidase and β -glucosidase from probiotic strains led to an increase in the concentration of isoflavone aglycones while a decrease in the concentration of isoflavone glycosides. The changes in the isoflavone profile occurred due to the hydrolytic action of the enzyme, which was responsible for the de-conjugation of the sugar moiety attached at position 7 of the A ring. The total detectable isoflavone concentration was 56.08 $\mu\text{g mL}^{-1}$ in the soymilk. Isoflavone compounds that were not detected included malonylglycitein and equol. Possible reasons for the absence of malonylglycitein could be due to the varietal orientation of the parent soybean from which SPI was extracted

----- Chapter 10.0 A comparison of exogenous and endogenous β -glucosidases (Chapter 8.0). The detection of equol appears to be very limited in an *in vitro* process; however it is readily detectable in an *in vivo* system from human fluids such as blood plasma and urine (Jenkins *et al.* 2002). Glycoside glycitin was hydrolysed, while both daidzin and genistin were significantly ($P < 0.01$) hydrolysed by the same concentration of exogenous β -glucosidase, leading to a significant reduction ($P < 0.01$) of total isoflavone glycosides. On the other hand, there was a significant increase ($P < 0.01$) in the concentration of isoflavone aglycones reaching a 5.37 fold increase within 30 min as a result of the rapid hydrolysis of isoflavone glycosides. There was a significant increase ($P < 0.01$) in the concentration of aglycone genistein while the concentration of daidzein and glycitein, however, were not significant (Figure 10.1). Acetyl- and malonylglycosides occurred in small quantities and there was no significant change in their concentration during the 4 h incubation.

With increased enzyme concentration, the same general trend of glycoside hydrolysis occurred; the difference owing to higher enzyme activity of 0.359 U mL^{-1} . There was a significant increase ($P < 0.01$) in the aglycone concentration and a significant decrease ($P < 0.01$) in the glycoside concentration. There was no significant decrease in glycoside glycitin concentration, while there was a significant increase ($P < 0.01$) in the concentration of all the individual aglycones. Glycosides daidzin and genistin were hydrolysed within 30 min of incubation, leading to a significant increase ($P < 0.01$) in the concentration of their respective aglycones. There was a similarity of glycitin hydrolysis in both cases (Figure 10.1 and 10.2), in which a non significant decrease of glycitin led to a non significant increase in the aglycone glycitein concentration. The possible reason for the relatively slower hydrolysis of glycitin in comparison to other glycosides such as daidzin and genistin is the occurrence of a methoxy group in carbon 7 of the A ring very close to the glycosidic bond in carbon 6 of the same ring. The proximity of the methoxy group to the glycosidic bond causes some resistance to the approaching hydrolysing enzyme causing a steric hindrance. In a previous study by Tsangalis *et al.* (2004), steric hindrance was reported as a

----- Chapter 10.0 A comparison of exogenous and endogenous β -glucosidases possible reason for the relative low concentration of aglycone glycitein from glicitin hydrolysis using endogenous β -glucosidase from *Bifidobacterium*. It was interesting to note that that the same effect appeared to occur with the use of exogenous β -glucosidase.

With the use of a higher enzyme activity of 0.575 U mL^{-1} , the same general trend occurred as in Figures 10.1 and 10.2 except a significant increase ($P < 0.01$) in the aglycone conjugate glycitein from hydrolysis of glycitin glycosides. This result was interesting because of the implication that a higher concentration of the enzyme could be more effective at overcoming the steric effect leading to a greater hydrolysis of such compounds. Glycosides daidzin and genistin were both significantly hydrolysed ($P < 0.01$) resulting into a significant increase ($P < 0.01$) in the concentration of respective aglycone conjugates, daidzein and genistein.

Through the years, genetic modifications and agronomic changes do occur leading to varietal modifications of the soybean hence alterations in their isoflavone profiles (Wang & Murphy, 1994a, 1994b). In addition, SPI extraction procedures contribute to the changes in the isoflavone profile leading to variations in the aglycone equivalent ratios. For example, SPI SUPRO 590 from which soymilk was manufactured had a pre-hydrolysis aglycone equivalent ratio of 0.89:1.0:0.11, for genistein:daidzein:glycitein, respectively. It has been postulated that in order to increase the biological activity of soymilk, the concentrations of the more estrogenic aglycone forms such as genistein have to be increased through enzyme hydrolysis while maintaining the aglycone equivalent ratio constituted in the native soybeans.

The aglycone equivalent ratio is based on the relative concentration of genistein and glycitein with respect to that of daidzein. Using this, any relative change in concentration of genistein and glycitein with regard to daidzein concentration in soymilk can be measured and monitored during incubation. In general, each isoflavone aglycone concentration increased with incubation time owing to hydrolytic action on respective glycosides. Therefore, even though the ratio of daidzein to genistein and to glycitein appeared to be constant, it was actually increasing along with that of

----- Chapter 10.0 A comparison of exogenous and endogenous β -glucosidases genistein and glycitein. The relative increase of each of the aglycones differed depending on the rate of hydrolysis of their respective glycosides. As shown in Table 10.1, there was relatively greater increase in genistein concentration than that of glycitein and possible reasons could be due to the steric hindrance already discussed. The aglycone equivalent ratio of 1.34:1.00:0.09 considered close to that of native soybeans was obtained after 4 h of incubation with exogenous β -glucosidase of activity of 0.288 UmL^{-1} . However, with increase in enzyme concentration, optimal ratios were achieved with shorter incubation times of 3 h with activity of 0.359 UmL^{-1} (Figure 10.2) and 2 h with activity of 0.575 UmL^{-1} (Figure 10.3), respectively. The results suggested that increasing enzyme concentration enabled the occurrence of the native-like aglycone ratios in soymilk within shorter incubation times and vice versa. These data are very important in the optimisation of processing of a soy beverage designed to have high aglycone concentrations and optimal aglycone equivalent ratios within a short incubation period.

Table 10.1 also shows the aglycone equivalent ratios obtained in soymilk for up to 4 h of fermentation using β -glucosidase from selected micro-organisms. The same trend of increase in aglycone equivalent ratios increasing with fermentation time occurred as with the exogenous enzymes. Similarly, the apparent steric hindrance in the hydrolysis of glycoside glycitein was also exhibited resulting in low glycitein concentrations. Interestingly, near native-like aglycone equivalent ratios were also attained within the short fermentation time of 4 h. As shown, *L. acidophilus* ATCC 4461, *B. animalis* ssp. *lactis* Bb12 and *L. casei* 2607 took 2 h, 3 h and 4 h, respectively to attain the native-like aglycone equivalent ratios. There was, however, a fundamental difference between the isoflavone profile of soymilk fermented with exogenous and endogenous enzymes. There was a slightly higher increase in aglycone genistein in the equivalent ratio with fermentation time for hydrolysis by β -glucosidases produced during fermentation with probiotic strains compared to that of hydrolysis by added β -glucosidase. The reason for this is not understood at this point in time but could suggest that there is higher preferential hydrolysis of

----- Chapter 10.0 A comparison of exogenous and endogenous β -glucosidases glycoside genistin by endogenous β -glucosidase than that by exogenous one. This could primarily be due to the different sources of the β -glucosidase enzyme.

The total aglycone concentration increments with incubation during fermentation are shown in Table 10.2. There were increments of over 5 fold within 15 min of hydrolysis with the exogenous enzyme, while only about 1.5 times with the micro-organisms within the same time period. After 4 h of incubation, the aglycone concentration in the soymilk increased to a high of 5.87 fold with enzyme of activity of 0.288 UmL^{-1} . Interestingly, there was 6.07 fold increment with 0.359 UmL^{-1} activity after 3 h and 5.94 fold increment only after 2 h with enzyme activity of 0.575 UmL^{-1} . There appeared to a relationship between the enzyme activity and the time taken to achieve highest increment of aglycone concentration in the soymilk. The higher the concentration of exogenous β -glucosidase, the shorter the time it took to attain highest aglycone concentration and *vice versa*. In comparison with the 3 selected micro-organisms, the level of aglycone increments for exogenous enzyme was much higher. This could suggest that exogenous enzymes had a higher rate of glycoside hydrolysis, resulting into higher rate of aglycone concentration. *B. animalis* ssp. *lactis* Bb12 had highest of 3.43 fold aglycone increment followed by *L. acidophilus* 4461 and *L. casei* 2607 with 3.04 fold and 2.72 fold increments, respectively after 4 h of fermentation of the soymilk. From these results, the implication is that in order to attain higher *in vitro* aglycone concentration in soymilk within a short time of 4 h, exogenous β -glucosidase should be preferred to micro-organisms. The fundamental difference therefore between soymilk hydrolysed by exogenous and endogenous β -glucosidase was the levels of isoflavone glycosides and aglycones due to the difference in the rate of isoflavone transformation. For improvement in the biological activity of soymilk, high aglycone levels and native-like aglycone equivalents could be critical in the delivery of health benefits to the consumer. Hence soymilk fermented with probiotic micro-organisms for a short period of up to 4 h may not be ideal as compared to that with exogenous β -glucosidase.

Figure 10.4 shows each of the 3 micro-organisms and their hydrolysing potential in soymilk during fermentation. All 3 micro-organisms did not attain an O – S index of more than 1 until after 4 h of fermentation. In contrast, the O – S index were much higher for exogenous enzymes than that of the micro-organisms. After 1 h of incubation, the O – S index was already more than 1 for the 3 enzyme solutions used for hydrolysing isoflavone glycosides in the soymilk. The other interesting observation was that within the first 2 h of incubation, the O – S index of enzyme solution with the activity of 0.537 UmL^{-1} was higher than that with 0.379 UmL^{-1} and 0.288 UmL^{-1} . At 3 h of incubation, highest index was attained with enzyme activity of 0.379 UmL^{-1} while at 4 h, enzyme activity of 0.288 UmL^{-1} had highest O – S index. However, as incubation progressed, exogenous enzyme with lower enzyme activity would show higher O – S index than that with higher enzyme activity. It is not fully understood why this is so at this point in time except that it is well known that enzyme activity decreases with a reduction in substrate concentration. This trend however is interesting because it reveals a direct correlation between the hydrolytic potential and the aglycone increment in soymilk during incubation (Figure 10.4 and Table 10.2). The higher the O – S index, the higher the aglycone increment could be and vice versa. The other interesting correlation was that the attainment of the “native soybean-like” aglycone equivalent ratio directly coincided with the highest O – S index (Figure 10.4 and Table 10.1).

Figure 10.5 shows the O – S indices of the 3 micro-organisms during extended 24 h fermentation. The hydrolytic potential (O – S index) of the micro-organisms reached peak values after 15 h of fermentation in the fermented soymilk, correlating with peak β -glucosidase activity (Chapter 9.0), just like in our earlier study (Chapter 4.0). There also appeared to be a linear relationship between the β -glucosidase activity and the aglycone concentrations. Extending the incubation time beyond 4 h for soymilk hydrolysed with exogenous enzymes was not necessary as there was no further major increment in aglycone concentrations. As for endogenous enzyme, it became

----- Chapter 10.0 A comparison of exogenous and endogenous β -glucosidases necessary to extend fermentation time since the aglycone concentration was lower than that for exogenous enzyme within the 4 h period. O-S indices appeared to follow the growth cycle of the micro-organisms. Interestingly, the total aglycone increment was also highest after 15 h for the three micro-organisms, implying that in order for soymilk to have improved biological activity, such a fermentation time would be required. The aglycone increment of *B. animalis* ssp. *lactis* Bb12, *L. acidophilus* ATCC4461 and *L. casei* 2607 after 15 h of fermentation in soymilk were 5.75 fold, 5.72 fold and 5.95 fold (Figures 10.6, 10.7 and 10.8), respectively, thus comparing favourably to the increment levels attained with exogenous β -glucosidase within approximately 2 h. The other important comparison between the exogenous and endogenous β -glucosidase was the aglycone-glycoside equilibrium cross over point. It took much shorter time with exogenous than with endogenous β -glucosidase to attain the equilibrium point. The time it takes to reach this point is an important indicator of the hydrolysing potential of the enzyme. The shorter the time it takes, the higher the hydrolysing potential and vice versa. The differences in the kinetics of hydrolysing strength could again be attributed to difference in the concentration of the enzyme source and the growth cycle of the microorganism.

10.5 CONCLUSIONS

Based on this study, two types of soymilk with improved biological activity can be manufactured. Soymilk whose isoflavone glycosides are hydrolysed with exogenous enzymes will have a very short incubation time at 37°C (at least 2 h), high aglycone concentration and ‘native soybean-like’ aglycone equivalent ratios. On the other hand, fermented soymilk with these lactic acid bacteria would have to undergo a longer fermentation time (approx. 15 h) in order to have highest aglycone concentration, optimum aglycone equivalent ratios, acidification by fermentation and at least 10^6 cfu per mL of probiotic micro-organisms based on 5% v/v inoculation. A combination of high *in vitro* aglycone concentration with optimal aglycone equivalent ratio could be the secret

----- *Chapter 10.0* A comparison of exogenous and endogenous β -glucosidases for full health potential of isoflavones in soymilk. The two types of soymilk could provide a critical beginning point for clinical trials in order to realise fuller health benefits of the unique functional attributes of soy isoflavones.

Table 10.1 Aglycone equivalent ratios for isoflavone aglycones in soymilk during Incubation/Fermentation period at 37°C with different concentrations of exogenous β -glucosidase and microorganisms

	0 (h)	0.25 (h)	0.5 (h)	1.0 (h)	2.0 (h)	3.0 (h)	4.0 (h)
0.288 UmL ⁻¹							
Genistein	0.89	0.99	0.98	1.04	1.13	1.33	1.34
Daidzein	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glycitein	0.11	0.03	0.03	0.04	0.04	0.06	0.09
0.359 UmL ⁻¹							
Genistein	0.89	0.96	1.12	1.22	1.23	1.28	1.30
Daidzein	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glycitein	0.11	0.03	0.04	0.04	0.03	0.05	0.05
0.575 UmL ⁻¹							
Genistein	0.89	1.09	1.24	1.14	1.35	1.42	1.41
Daidzein	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glycitein	0.11	0.05	0.08	0.07	0.07	0.07	0.07
Bb12							
Genistein	0.89	1.09	1.21	1.12	1.25	1.34	1.37
Daidzein	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glycitein	0.11	0.01	0.02	0.06	0.04	0.04	0.04
LC 2607							
Genistein	0.89	1.16	1.24	1.27	1.26	1.28	1.34
Daidzein	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glycitein	0.11	0.06	0.06	0.07	0.05	0.05	0.06
LA 4461							
Genistein	0.89	1.24	1.05	0.88	1.32	1.35	1.37
Daidzein	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glycitein	0.11	0.06	0.06	0.07	0.07	0.05	0.06

Bb12 – *Bifidobacterium animalis* ssp. *lactis* Bb12

LC 2607 – *Lactobacillus casei* 2607

LA 4461 – *Lactobacillus acidophilus* ATCC4461

Aglycone Equivalent Ratio – Is the ratio of concentration of Genistein: Daidzein: Glycitein in soymilk using aglycone daidzein as reference.

Bolded Values – Are points of optimum aglycone equivalent ratios during incubation or fermentation of soymilk with exogenous β -glucosidase or micro-organism

Table 10.2 Increments of total aglycone concentration in soymilk during 4h incubation with different concentrations of exogenous β -glucosidase and micro-organisms

	0 (h)	0.25 (h)	0.5 (h)	1.0 (h)	2.0 (h)	3.0 (h)	4.0 (h)
0.288 UmL ⁻¹	1	5.30	5.35	5.70	5.80	5.83	5.87
0.359 UmL ⁻¹	1	5.18	5.67	5.85	6.04	6.07	5.92
0.575 UmL ⁻¹	1	5.58	5.63	5.84	5.94	5.65	5.77
Bb12	1	1.49	1.33	2.14	2.45	3.14	3.43
LC 2607	1	1.33	1.44	1.55	2.22	2.79	2.72
LA 4461	1	1.62	1.51	1.55	2.61	3.01	3.04

Bb12 – *Bifidobacterium animalis* ssp. *lactis* Bb12

LC 2607 – *Lactobacillus casei* 2607

LA 4461 – *Lactobacillus acidophilus* ATCC4461

Table values – Number of times increment of the initial aglycone concentration in soymilk

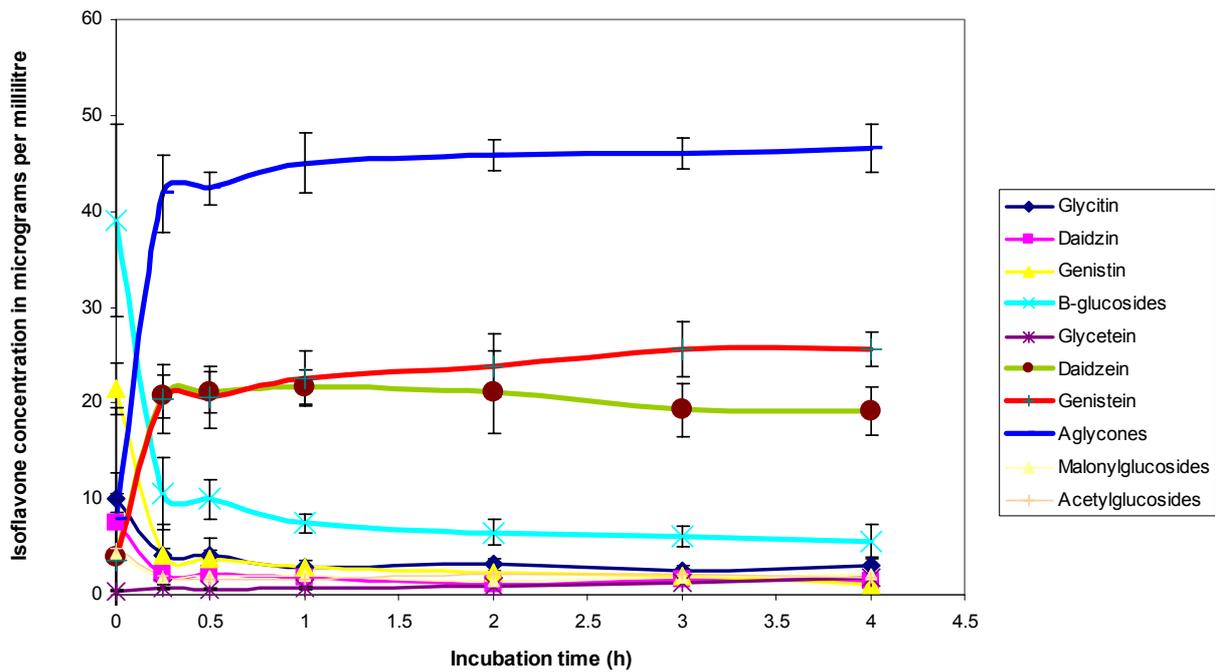


Figure 10.1 Changes in isoflavone concentration in soymilk during incubation at 37°C using 0.288 U mL^{-1} of exogenous β -glucosidase. Results expressed as mean \pm standard deviation (SD) ($n = 6$). One unit of the enzyme activity was defined as the amount of β -glucosidase that released one micromole of p -nitrophenol from p NPG per mL per min under assay conditions

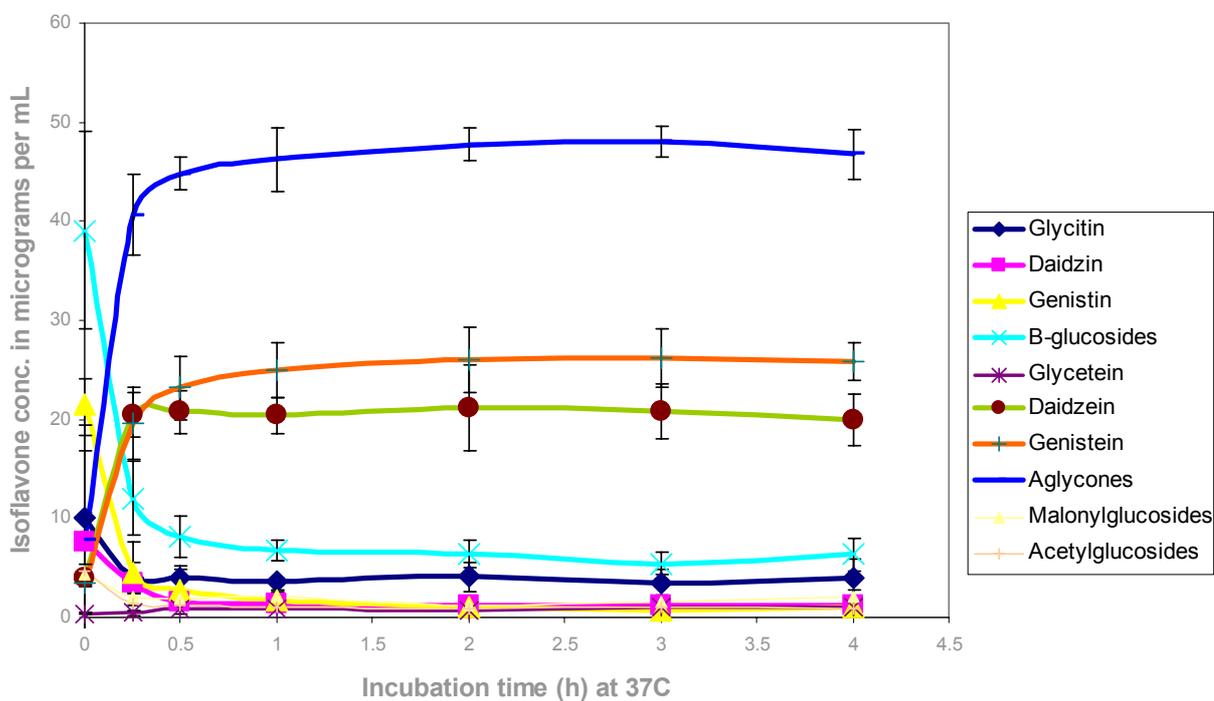


Figure 10.2 Changes in isoflavone concentration in soymilk during incubation at 37°C using 0.359 U mL^{-1} of exogenous β -glucosidase. Results expressed as mean \pm standard deviation (SD) ($n = 6$). One unit of the enzyme activity was defined as the amount of β -glucosidase that released one micromole of p -nitrophenol from p NPG per mL per min under assay conditions

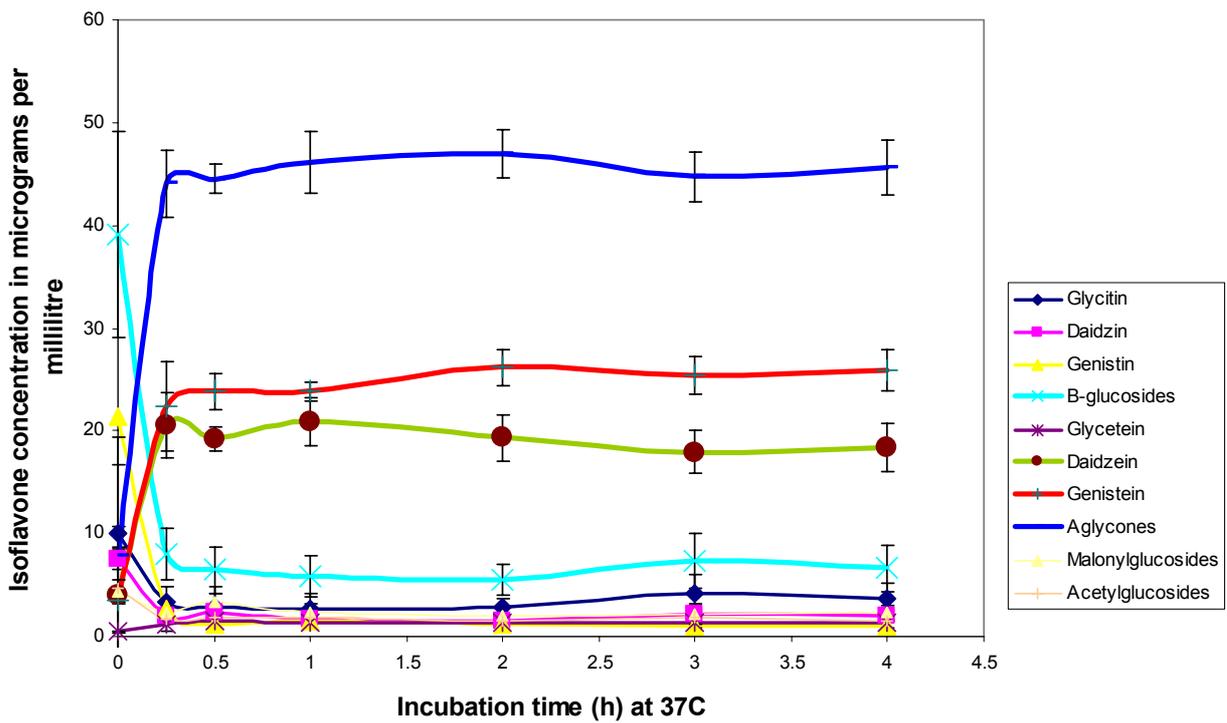


Figure 10.3 Changes in isoflavone concentration in soymilk during incubation at 37°C using 0.575 U mL⁻¹ of exogenous β -glucosidase. Results expressed as mean \pm standard deviation (SD) (n = 6). One unit of the enzyme activity was defined as the amount of β -glucosidase that released one micromole of p-nitrophenol from pNPG per mL per min under assay conditions

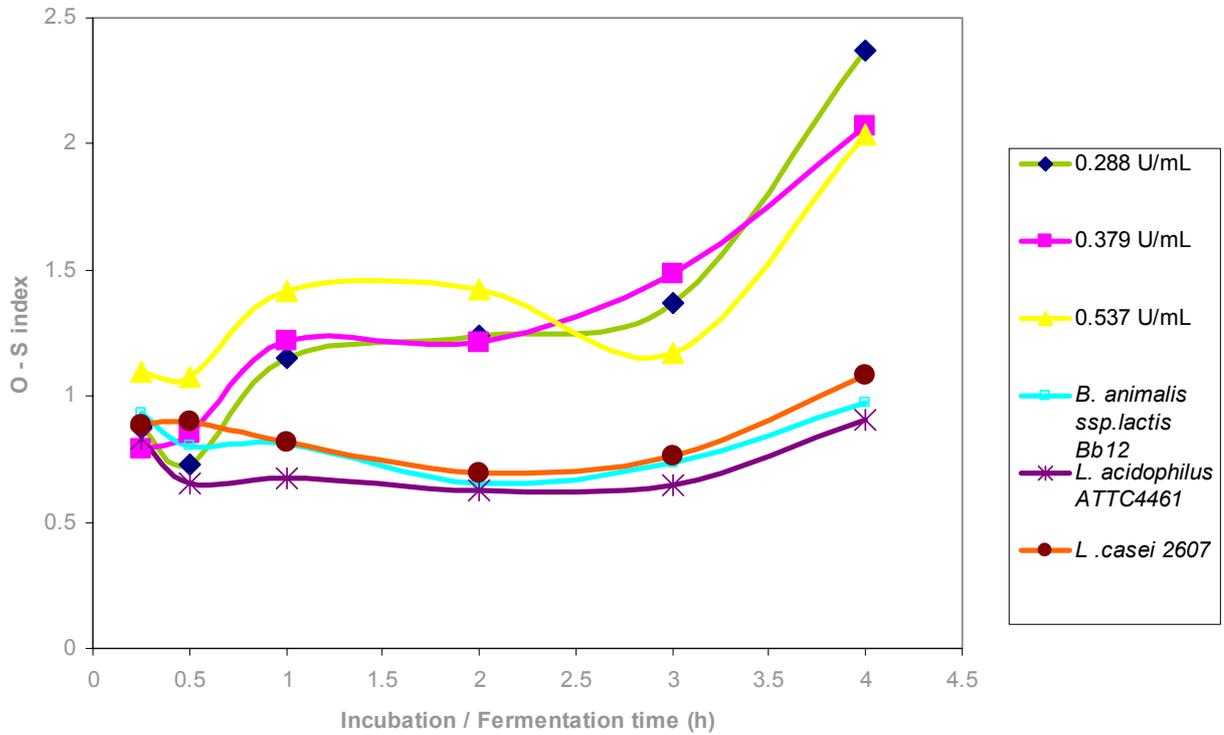


Figure 10.4 Comparison of hydrolytic potential (Otiemo Shah (O-S) index) of probiotic micro-organisms and exogenous β -glucosidase in isoflavone transformation in the soymilk during 4 h fermentation/incubation at 37°C.

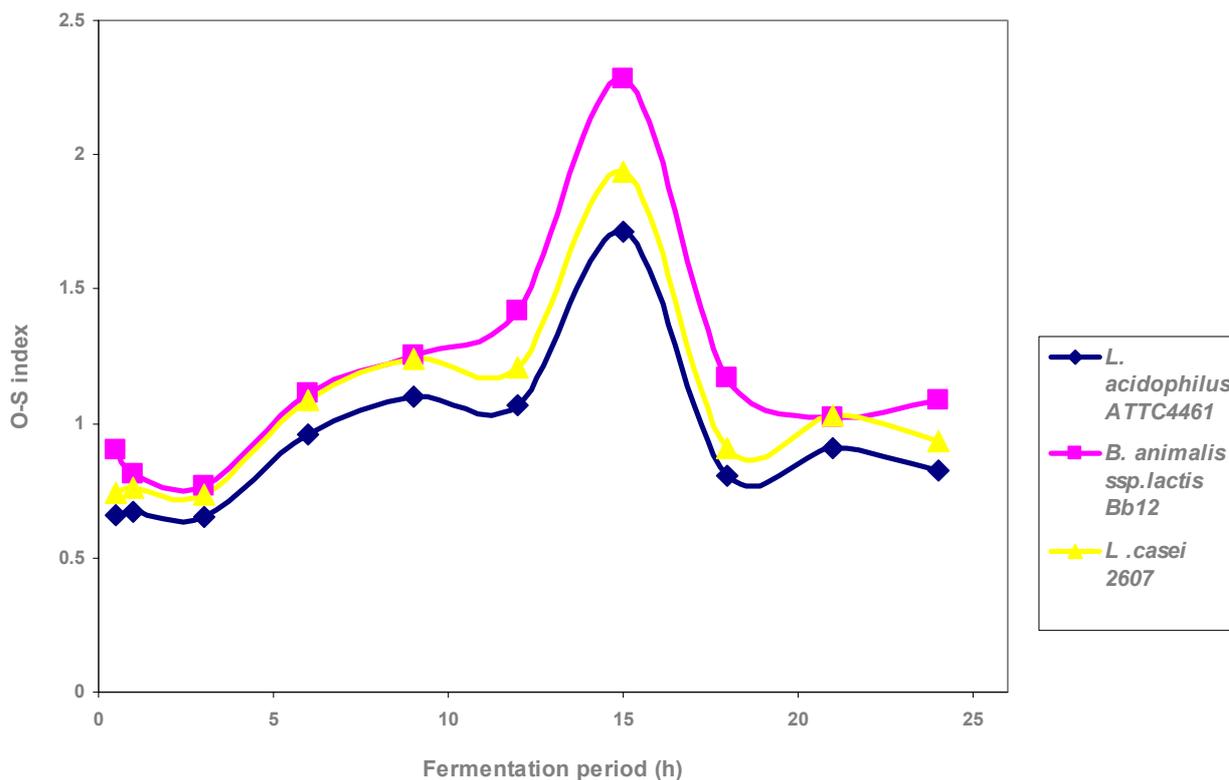


Figure 10.5. Hydrolytic indices (Otieno Shah (O-S) indices) of isoflavone hydrolysis in soymilk using probiotic micro-organisms during 24 h fermentation at 37°C.

Otieno-Shah (O-S) index (isoflavone hydrolytic index)- refers to the ratio of β -glycoside reduction quotient to the aglycone increment quotient.

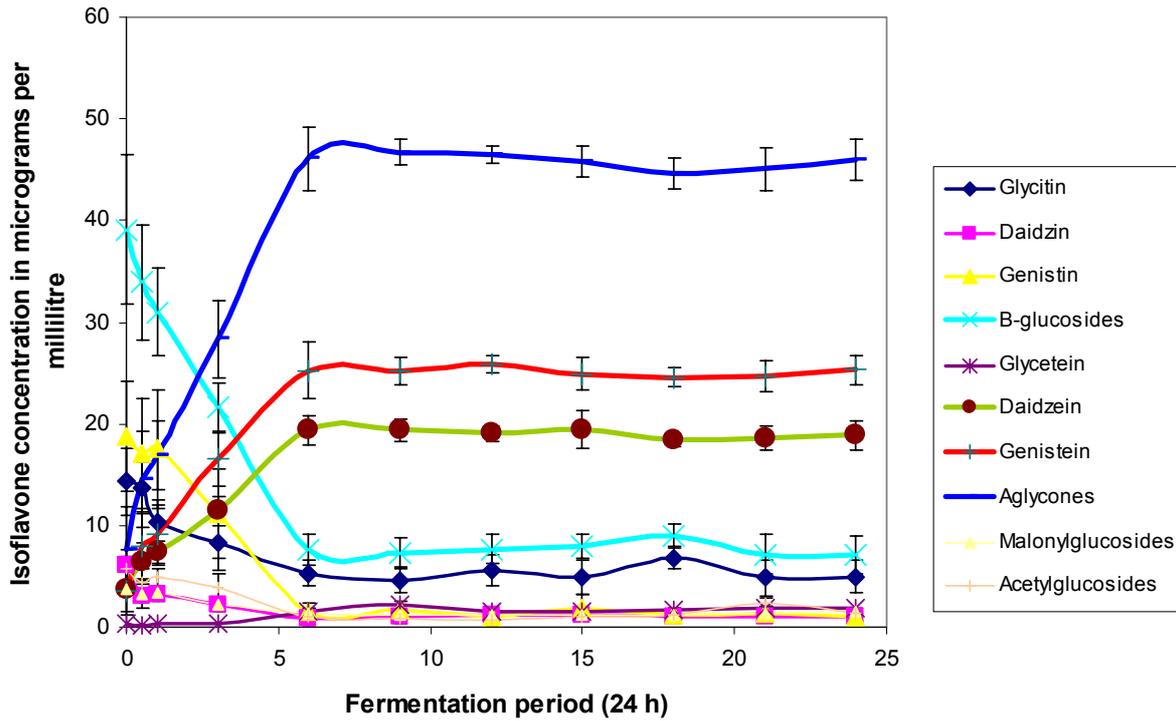


Figure 10.6 Changes in isoflavone concentration ($\mu\text{g/mL}$) in soymilk during fermentation with *Bifidobacterium animalis ssp. lactis* Bb12. Results expressed as mean \pm standard deviation (SD) in $\mu\text{g/mL}$ of fermented soymilk ($n = 6$).

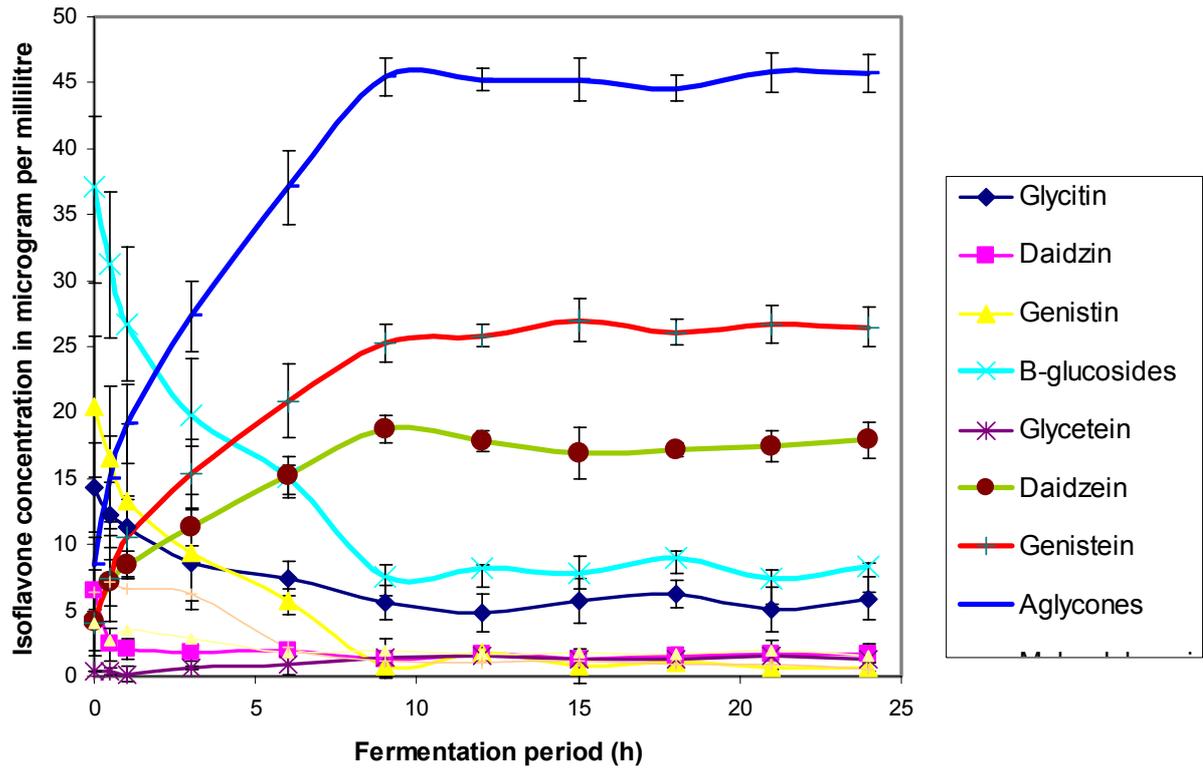


Figure 10.7 Changes in isoflavone concentration ($\mu\text{g/mL}$) in soymilk during fermentation with *Lactobacillus acidophilus* ATCC4461. Results expressed as mean \pm standard deviation (SD) in $\mu\text{g/mL}$ of fermented soymilk ($n = 6$).

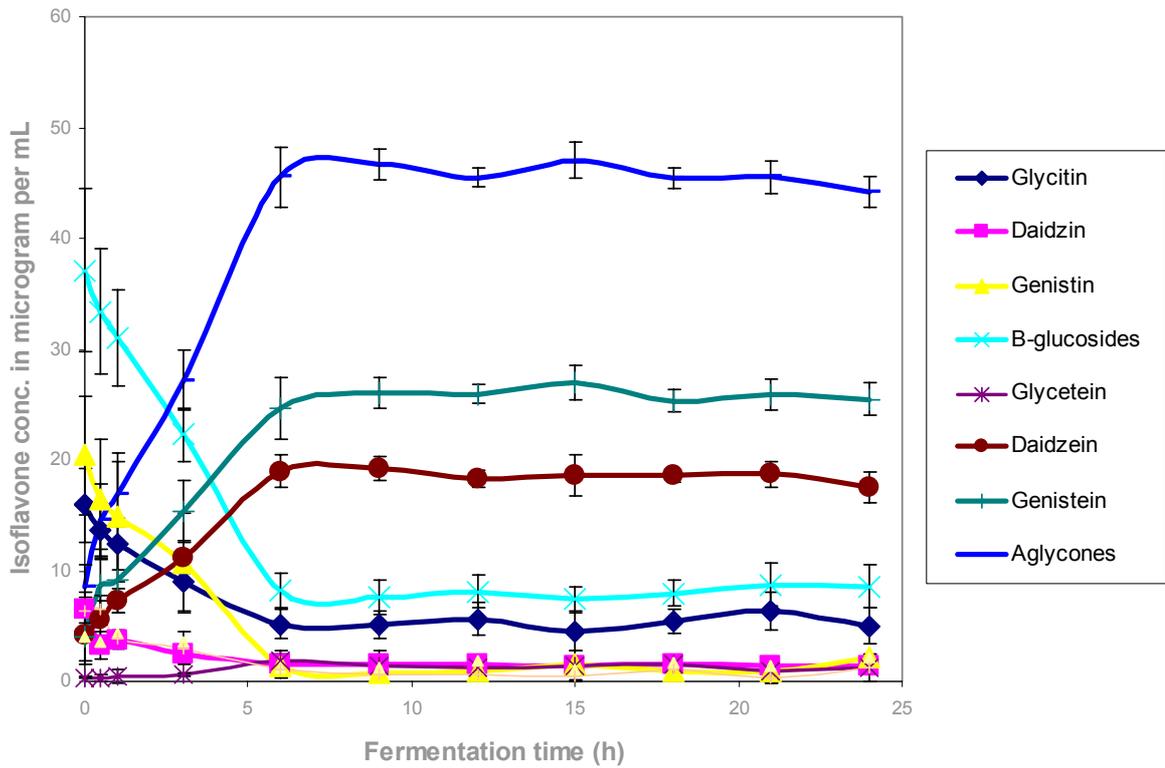


Figure 10.8. Changes in isoflavone concentration ($\mu\text{g/mL}$) in soymilk during fermentation with *Lactobacillus casei* 2607
Results expressed as mean \pm standard deviation (SD) in $\mu\text{g/mL}$ of fermented soymilk ($n = 6$).

11.0 Overall Conclusions and Future Research Direction

This research showed that in addition to *Bifidobacterium*, other probiotic microorganisms including *L. acidophilus* 33200, *L. casei* 2607, *L. acidophilus* 4962, *L. acidophilus* 4461 and *L. casei* ASCC 290 produced varying levels of β -glucosidase activity that may play an important role in improving the biological activity of soymilk. While the enzyme activity is critical in the biotransformation of predominant isoflavone glycosides to the bioactive isoflavone aglycones in soymilk, its stability at different storage temperatures influenced the hydrolysing potential. We found that stability of the enzyme varied at different storage temperatures and declined over time. Storage at 4°C for up to 5 weeks for maintaining appreciable levels of β -glucosidase activity and better stability of the enzyme would be ideal as was observed from storage studies. A correct storage temperature is therefore important for maintaining high level of β -glucosidase activity from probiotic microorganisms as well as for its stability in soymilk.

Evaluating the enzymic potential of the selected bacteria for its role in isoflavone biotransformation is an important step in the development of aglycone rich soy based foods. Since it was established in the study that six microorganisms produced varying levels of β -glucosidase activity for enzyme-induced biotransformation of the isoflavone β -glycosides depending on the stage of fermentation, it was possible to evaluate their hydrolytic potential. This was found to be unique for each strain. A novel method for precise evaluation of the hydrolysing potential of isoflavone glycosides, for any microorganism was developed and the index of measurement proposed as Otieno - Shah (O – S) index. This evaluation process has been appreciated and published in international journals of repute as versions of Chapters 3.0, 9.0 and 10.0. Effective and precise selection criteria for probiotic microorganisms for use in improving the biological activity of soymilk has therefore been established and can be of use in the development of aglycone rich probiotic soy based foods.

It was important to find out the stability of isoflavone glycosides and aglycones under various storage temperatures. The possible effects of the changes in isoflavones structure and composition on their bioactivity during storage may affect bioavailability and biological activity. A comprehensive study on the stability of isoflavones during storage as influenced by probiotic microorganism was conducted and we found that the isoflavone glycosides and aglycones were in general stable in soymilk during storage at -80°C, 4°C, 25°C and 37°C. However, using first order kinetics we found that the relative stability of isoflavone compounds differed at different storage temperatures. Isoflavone aglycones were observed as more stable compared to glycosides. Of the isoflavone aglycones, daidzein was most stable followed by genistein, while glycitein was least stable. The stability of the isoflavone aglycones in the soymilk could be primarily influenced by their molecular configurations, the storage temperatures as well as the hydrolysing influence of the microbial enzyme on isoflavone glycosides. We also found that these probiotic microorganisms had different preferential hydrolytic affinity to certain isoflavone glycosides, especially genistin in fermented soymilk. This ability could be important in targeting specific isoflavone aglycones and their metabolites. Storage temperature, however, played the most influential role in regulating the rate of degradation of enzyme – induced soy isoflavones in fermented soymilk. For better stability of the isoflavone aglycones of daidzein, glycitein and genistein in fermented soymilk, lower storage temperatures are recommended. A linear model such as first order kinetics is important in determining the rate of degradation of isoflavone compounds in fermented soymilk and can therefore be used as a guide in determining best storage conditions for a product containing isoflavone glycosides and aglycones. Therefore, for optimum stability of the bioactive isoflavone aglycones of daidzein, glycitein and genistein in fermented soymilk, lower storage temperatures, particularly 4°C is most suitable.

It is critically important to establish the total content and profile of compounds whose concentration and composition are critical in human health. Using HPLC and LC-MS/MS

(Extracted ion chromatography and positive ion fragmentation techniques), we were able to establish the isoflavone content and profile of SPI, unfermented soymilk and fermented soymilk. We found that there was no similarity in the fragment product ions, indicating the uniqueness of each isoflavone compound. Although there were differences in the spectra of product ions from individual isoflavones in soymilk, the fragmentation patterns of classes of isoflavones (i.e. aglycones, glycosides, acetyl-, and malonyl- forms) followed a similar trend. There was no similarity of particular peaks amongst the isoflavone aglycones but there was a similarity in the trend of loss of carbon monoxide, at least successively with daidzein and genistein. All isoflavone glycosides including the malonyl and acetyl forms detected in soymilk had respective aglycone ions as major peaks in the spectra. Glycosides such as daidzin and glycitin had a similar fragment ion of m/z 206 while genistin, acetylgenistin, acetylglycitin, malonyldaidzin and malonylgenistin had a common occurrence of fragment ion of 184 m/z ($C_{12}H_8S$). Eventhough there were similarities in the fragments amongst the isoflavone glycosides and in the trend of losses of ions and molecules during fragmentation of each class of isoflavones, each compound, however, had a unique fragmentation pattern leading to their unequivocal identification. The identification of isoflavones in fermented and unfermented soymilk is necessary to generate information for *in vivo* and *in vitro* studies and for the authentication of isoflavones in soy based drinks and beverages.

In actuating the enzymic hydrolysis of isoflavones in soymilk, it was important to assess which enzyme hydrolases among the crude endogenous enzymes in probiotic bacteria played a greater role in isoflavone biotransformation. We found that β -glucosidase activity was higher than β -galactosidase activity in soymilk for all the probiotic microorganisms studied and would impact a higher aglycone increment value in soymilk comparatively. There was also a similar trend of increase in β -glucosidase activity, β -galactosidase activity and aglycone increment value during fermentation all reaching peak at 15 h of fermentation. It would be reasonable to allow 15 h

fermentation time to substantially improve the biological activity of soymilk with these probiotic microorganisms.

We finally compared the rate and effectiveness of isoflavone biotransformation between exogenous and endogenous β -glucosidase. Based on this aspect of study, two types of soymilk with improved biological activity can be manufactured. Soymilk whose isoflavone glycosides is hydrolysed with exogenous enzymes will have a very short incubation time of 2 h at 37°C, high aglycone concentration and “native soybean-like” aglycone equivalent ratios. On the other hand, fermented soymilk with these probiotic bacteria would have to undergo a longer fermentation time (approx. 15 h) in order to have highest aglycone concentration, optimum aglycone equivalent ratios, acidification by fermentation and at least 10^6 cfu per millilitre of probiotic micro-organisms based on 5% (v/v) inoculation. A combination of high *in vitro* aglycone concentration with optimal aglycone equivalent ratio could be the secret for full health potential of isoflavones in soymilk. The two types of soymilk could provide a critical beginning point for clinical trials in order to realise fuller health benefits of the unique functional attributes of soy isoflavones.

With regards to developing this research into the future, it would be worthwhile to investigate the hydrolytic ability of alternative and cheaper sources of exogenous β -glucosidase on isoflavone glycosides apart from the almond extracted β -glucosidase that has been extensively used in our *in vitro* studies. It should not only be necessary but also inevitable to evaluate the role of exogenous β -galactosidase in isoflavone biotransformation, and make comparison with that of exogenous β -glucosidase. Along with this, a further investigation into the profile of possibly different sugar constituents of the predominant isoflavone glycosides in soymilk. The crowning of these further investigations should then be a confirmation of the efficacy of the soymilk with the above attributes through conducted intervention clinical dietary trials. There are four main

areas of clinical trials that may be investigated for the potential health benefits to the consumers. This can be assessed by observing and analysing the trends of specific and relevant biomarkers before, during and at the end of the intervention study period. These will include:-

1. Intervention study involving the use of the soymilk and its consumption effects on cardiovascular health of a selected study group (population).
2. Intervention study involving the use of the soymilk and its consumption effects on bone loss prevention among post menopausal women. This will involve study of the possible relationship between calcium absorption and soymilk consumption.
3. Intervention study involving the use of the soymilk and its consumption effects on the lipid profiles of postmenopausal women.
4. Intervention study involving use of the soymilk and its consumption effects on relief of post-menopausal symptoms such as hot flushes, and osteoporosis.

12.0 List of References

- Adlercreutz CHT, Goldin BR & Gorbach SL (1995) Soybean phytoestrogen intake and cancer risk. *J Nutr* **125**, 757S-770S.
- Adlercreutz H (1998) *Human health and phytoestrogens*. In: Korach, KS, ed. Reproductive and Developmental Toxicology. New York, NY: Marcel Dekker, Inc. p299-371.
- Adlercreutz H, Gorbach SL, Goldin BR, Woods MN, Dwyer JT & Hamalainen E (1994) Estrogen metabolism and excretion in Oriental and Caucasian women. *J Natl Cancer Inst* **86**, 1076-1082.
- Adlercreutz H, Hamalainen E, Gorbach S & Goldin B (1992) Dietary phytoestrogens and the menopause in Japan (Letter). *Lancet* **339**, 1233.
- Albright SC, Winston WL & Zappe C (1999) *Data Analysis and Decision Making with Microsoft Excel*. Pacific Grove, CA: Brooks/Cole Publishing Co.
- Alice LM, Wilcox G & Davis SR (1998) Phytoestrogens, clinical review 92. *J Clin Endocrinol Metab* **83**, 2, 297-303.
- Allred CD, Ju YH, Allred KF, Chang J & Helferich WG (2001) Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein, *Carcinogenesis* **22**, 1667-1673.
- American Soybean Association & United Soybean Board. Designer soybeans (2001). <http://www.asajapan.org/english/food/quality/02.html> (Accessed on 20th January, 2007)
- Anderson JJB & Garner SC (1997) Phytoestrogens and human function. *Nutr Today* **32**, 232-239.
- Anderson JJB & Garner SC (1998) Phytoestrogens and bone, Baillieres *Clin Endocrinol Metab* **12**, 543-557.
- Anderson JW, Johnstone BM & Cook-Newell ME (1995) Meta-analysis of the effects of soy protein intake on serum lipids, *N Engl J Med* **333**, 276-282.

- Anthony MS, Clarkson TB, Hughes CL Jr, Morgan TM & Burke GL (1996) Soybean isoflavones improve cardiovascular risk factors without affecting the reproductive system of peripubertal rhesus monkeys. *J Nutr* **126**, 43-50.
- Arjmandi BH, Alexkel L, Hollis BW, Amin D, Stacewiczsapunzakis M, Guo P & Kukreja SC (1996) Dietary soybean protein prevents bone loss in an ovariectomised rat model of osteoporosis. *J Nutr* **126**, 161-167.
- Ashton EL, Dalais FS & Ball MJ (2000) Effect of meat replacement by tofu on CHD risk factors including copper induced LDL oxidation. *J Am Coll Nutr* **19**, 761-767.
- Atanassova N, McKinnell C & Turner KJ (2000) Comparative effects of neonatal exposure of male rats to potent and weak (environmental) estrogens on spermatogenesis at puberty and the relationship to adult testis size and fertility: evidence for stimulatory effects of low estrogen levels. *Endocrinology* **141**, 3898-3907.
- Axelsson L (1998). *Lactic Acid Bacteria: Classification and Physiology*. In: Salminen S, Von Wright A, editors. *Lactic Acid Bacteria*. New York : Marcel Dekker. 1 - 72p.
- Ballongue J. 1993. *Bifidobacteria and probiotic action*. In : Salminen S, Von Wright A, editors. *Lactic Acid Bacteria*. New York : Marcel Dekker. 519 –587p
- Barnes S, Grubbs C, Setchell KDR & Carlson J (1990) Soybeans inhibit mammary tumors in models of breast cancer *Prog Clin Biol Res* **347**, 239-253.
- Barnes S, Kirk M & Coward L (1994) Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-mass spectrometry. *J Agric Food Chem* **42**, 2466 – 2474.
- Barrett-Connor E (1997) Sex differences in coronary heart disease. Why are women so superior? The 1995 Ancel Keys Lecture. *Circulation* **95**, 252–64.
- Bayer T, Colnot T & Dekant W (2001) Disposition and biotransformation of the estrogenic isoflavone daidzein in rats. *J Toxicol Sci* **62**, 205 - 211.

- Berrada N, Lemeland JF, Laroche G, Thouvenot P & Piaia M (1991). *Bifidobacterium* from fermented milks: Survival during gastric transit. *J Dairy Sci* **74**, 409-413.
- Boersma BJ, Barnes S & Kirk M (2001) Soy isoflavonoids and cancer-metabolism at the target site. *Mutat Res* **480**, 121-127.
- Bradlow HL, Fishman J, Telang NT & Osborne MP (1994) Estrogen metabolism and excretion in Oriental and Caucasian women. *J Natl Cancer Inst* **86**, 1643-1645.
- Bradlow HL, Fishman J, Telang NT, Osborne MP (1994) Estrogen metabolism and excretion in Oriental and Caucasian women. *J Natl Cancer Inst* **86**, 1643–1645.
- Bruno FA & Shah NP (2003) Viability of two freeze dried strains of *Bifidobacterium* and commercial preparations at various temperatures during prolonged storage. *J Food Sci* **68**, 7, 2336–2339.
- Buttris J (1997) Nutritional properties of fermented milk products. *Int J Dairy Tech* **50** (1), 21-27.
- Casanova M, You L, Gaido KW, Archibeque-Engle S, Janszen DB & Heck H (1999) Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein with rat estrogen receptors and in vitro. *Toxicol Sci* **51**, 236-244.
- Cassidy A (1996) Physiological effects of phyto-oestrogens in relation to cancer and other human health risks. *Proc Nutr Soc* **55**, 399 - 417.
- Cassidy A, Hanley B & Lamuela-Raventos, R. M (2000). Isoflavones, lignans and stilbenes: Origins, metabolism and potential importance to human health. *J Sci Food Agric* **80**, 1044-1062.
- Chen G, Zhang J, & Ye J (2001) Determination of puerarin, daidzein and rutin in *Pueraria lobota* (wild) *Ohwi* by capillary electrophoresis with electrochemical detection. *J Chromatogr A* **923**, 255 – 262.
- Chen Z, Zheng W, Custer LJ, Dai Q, Shu XO & Jin F (1999) Usual dietary consumption of soy foods and its correlation with the excretion rate of isoflavonoids in overnight urine samples among Chinese women in Shanghai. *Nutr Cancer* **33**, 82 - 87.

- Chiechi L, Secreto G, D'Amore M, Fanelli M, Venturelli E, Cantatore F, Valerio T, Laselva G & Loizzi P (2002) Efficacy of a soy rich diet in preventing postmenopausal osteoporosis: the Menfis randomized trial. *Maturitas* **42**, 495.
- Clarkson TB (2002) Soy, soy phytoestrogens and cardiovascular disease *J Nutr* **132**, 566S-569S.
- Clifton-Bligh PB, Baber RJ, Fulcher GR, Nery M & Moreton T (2001) The effect of isoflavones extracted from red clover (Rimostil) on lipid and bone metabolism. *Menopause* **8**, 4, 259-265.
- Cohen LA, Zhao Z, Pittman B & Scimeca JA (2000) Effect of intact and isoflavone-depleted soy protein on NMU-induced rat mammary tumorigenesis, *Carcinogenesis* **21**, 929-935.
- Constantinou AI, Lantvit D, Hawthorne M, Xu XY, van Breemen RB & Pezzuto JM (2001) Chemopreventive effects of soy protein and purified soy isoflavones on DMBA-induced mammary tumors in female Sprague-Dawley rats *Nutr.Cancer* **41**, 75-81.
- Cooper C & Melton LJ III (1992) Epidemiology of osteoporosis. *Trends Endocrinol Metab* **3**, 224-229.
- Cooper C, Campion G & Melton LJ III (1992) Hip fractures in the elderly: a world-wide projection *Osteoporos Int* **2**, 285-289.
- Coward L, Smith M, Kirk M & Barnes S (1998) Chemical modification of soy foods during cooking and processing. *Am J Clin Nutr* **68**, 1486S-91S.
- Dai Q, Franke AA, Jin F, Shu XO, Hebert JR, Custer LJ, Cheng JR, Gan YT & Zheng W (2002) Urinary excretion of phytoestrogens and risk of breast cancer among Chinese women in Shanghai *Cancer Epidemiol Biomarkers Prev* **11**, 815-821.
- Dalais F S, Wahlqvist ML, & Rice GE (1997) Isoflavonoid phytoestrogen content of soy food products in the Australian market. *Proc Nutr Soc Australia* **21**, 123.

- Dave RI & Shah NP (1996) Evaluation of media for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, and Bifidobacteria. *J Dairy Sci* **79**, 1529-1536.
- Davies CGA, Netto FM, Glassenap CM, Gallaher CM, Labuza TP & Gallaher DD (1998) Indication of the Maillard reaction during storage of protein isolates. *J Agric Food Chem* **46**, 2485–2489.
- Davis SR (2001) Phytoestrogen therapy for menopausal symptoms?. There's no good evidence that it's any better than placebo *Br Med J* **323**, 354-355.
- de Kleijn MJ, van der Schouw YT & Wilson PW (2001) Intake of dietary phytoestrogens is low in postmenopausal women in the United States: the Framingham study. *J Nutr* **131**, 1826 - 1832.
- de Kleijn MJ, van der Schouw YT, Wilson PW, Grobbee DE & Jacques PF (2002) Dietary intake of phytoestrogen is associated with a favourable metabolic cardiovascular risk profile in postmenopausal US. Women: the Framingham study. *J Nutr* **132**, 276.
- de la Rochefordiere A, Clough K, Fourquet A & Magdelenat H (2001) Phytoestrogens after breast cancer (The Breast Cancer Group of the Institute Curie). *Endocr Relat Cancer* **8**, 129-134.
- de Lemos M (2002) Safety issues of soy phytoestrogens in breast cancer patients *J Clin Oncol* **20**, 3040-3041.
- de Mann JC, Rogosa M & Sharpe ME (1960) A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**,130-135.
- De Vries RP, van den Broek HC, Dekkers E, Manzanares P, de Graff LH & Visser J (1999) Differential expression of three α -galactosidase genes and a single β -galactosidase gene from *Aspergillus niger*. *Appl Environ Microbiol* **65**, 2453 – 2460.

- den Tonkelaar I, Keinan-Boker L, Van't Veer P, Arts CJM, Adlercreutz H, Thijssen JHH & Peeters PHM (2001) Urinary phytoestrogens and postmenopausal breast cancer risk *Cancer Epidemiol Biomarkers Prev* **10**, 223-228.
- Desjardins ML & Roy D (1990) Growth of bifidobacteria and their enzyme profiles. *J Dairy Sci* **73**, 299 – 307.
- Dhom G (1991) *Epidemiology of hormone-dependent tumors*. In: Voigt KD, Knabbe C, eds. Endocrinedependent Tumors. New York: Raven Press p1-42.
- Doerge DR, Chang HC, Churchwell MI & Holder CL (2000) Analysis of soy isoflavones conjugation in vitro and in human blood using liquid chromatography mass spectrometry. *Drug Metab Dispos* **28**, 298 - 307.
- Dotzel MM (1999) Food labeling: Health claims; Soy protein and coronary heart disease; final rule, *Federal Register* **64**, 57700-57733.
- Drane HM, Patterson DSP, Roberts BA & Saba N (1980) Oestrogenic activity of soya-bean products. *Food Cosmet Toxicol* **18**, 425-427.
- Driessen FM & De Boer R (1989) Fermented milks with selected intestinal bacteria: a healthy trend in new products. *Neth Milk Dairy J* **43**, 367-382.
- Duncan AM, Merz-Demlow BE, Xu X, Phipps WR & Kurzer MS. (2000) Premenopausal equol excretors show plasma hormone profiles associated with lowered risk of breast cancer. *Cancer Epidemiol Biomarkers Prev*, **9**, 581-586.
- Eden JA (2001) Managing the menopause: phyto-oestrogens or hormone replacement therapy? *Ann Med* **33**, 4-6.
- Eissen B, Ungar Y & Shimoni E (2003) Stability of isoflavones in soy milk stored at elevated and ambient temperatures. *J Agric Food Chem* **51**, 2212–2215.
- Elkind-Hirsch K (2001) Effect of dietary phytoestrogens on hot flushes: can soy-based proteins substitute for traditional estrogen replacement therapy? *Menopause* **8**, 154-156.

- Erdman JW (2000) Soy protein and cardiovascular disease - A statement for healthcare professionals from the Nutrition Committee of the AHA, *Circulation* **102**, 2555-2559.
- Faber KA & Hughes CL Jr (1993) Dose-response characteristics of neonatal exposure to genistein on pituitary responsiveness to gonadotropin releasing hormone and volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in postpubertal castrated female rats. *Reprod Toxicol* **7**, 35-39.
- Faure ED, Chantre P & Mares P (2002) Effects of a standardized soy extract on hot flushes: a multicenter, double-blind, randomized, placebo-controlled study *Menopause* **9**, 329-334.
- Fekete E, Karaffa L, Sandor E, Seiboth B, Biro S & Szentirmai C (2002) Regulation of formation of the intracellular β -galactosidase activity of *Aspergillus nidulans* *Arch Microbiol* **179**, 7 – 14.
- Ferguson SA, Jackson MM & Newbold RR (1998) Chronic developmental genistein treatment results in decreased body weight, but few behavioral alterations in rats [abstract]. *Neurotoxicol Teratol* **20**, 365.
- Fiedurek J & Szczodrak J (1994) Selection of strain, culture conditions and extraction procedures for optimum production of β -galactosidase from *Kluyveromyces lactis*. *Acta Microbiol Pol* **43**, 57 – 65.
- File SE, Jarrett N, Fluck E, Duffy R, Casey K & Wiseman H (2001) Eating soya improves human memory. *Psychopharmacology* **157**, 430-436.
- Flynn KM, Ferguson SA, Delclos KB & Newbold RR (2000) Multigenerational exposure to dietary genistein has no severe effects on nursing behavior in rats. *Neurotoxicology* **21**, 997-1001.
- Food and Agriculture Organisation of the United Nations and World Health Organisation. 2001, posting date. Regulatory and clinical aspects of dairy probiotics. FAO and WHO Expert Consultation Report. FAO and WHO working group report. (Online)

-
- Food and Drug Administration (1999) www.fda.gov/bbs/topics/ANSWERS/ANS00980.html
(accessed on 20th January 2007)
- Franke, A. A., Custer, L. J., Wang, W., & Shi, C. Y. (1998). HPLC analysis of isoflavonoids and other phenolic agents from foods and from human fluids. *Proc Soc Exper Biol Med* **217**, 263–273.
- Fujimaki M, Arai S, Kirigaya N, & Sakurai Y (1965) Studies of flavour compounds in soybeans. Part 1. Aliphatic carbonyl compounds. *Agric Biol Chem* **29**, 855 -858.
- Fuller R (1989) Probiotics in man and animals. *J Appl Bacteriol* **66**, 5, 365-378.
- Gallo D, Cantelmo F & Distefano M (1999) Reproductive effects of dietary soy in female Wistar rats. *Food Chem Toxicol* **37**, 493-502.
- Gallo D, Giacomelli S, Cantelmo F, Zannoni GF, Ferrandina G, Fruscella E, Riva A, Morazzoni P, Bombardelli E, Mancuso S, & Scambia G (2001) Chemoprevention of DMBA-induced mammary cancer in rats by dietary soy, *Breast Cancer Res Treat* **69**, 153-164.
- Gao YH & Yamaguchi M (1999) Suppressive effect of genistein on rat bone osteoclasts: apoptosis is induced through Ca²⁺ signaling. *Biol Pharm Bull* **22**, 805–809
- Gao YH & Yamaguchi M (1999) Synergistic effect of genistein and casein phosphopeptides on bone components in young and elderly female rats. *Biochem Pharm*, **58**, 767.
- Gayer J (2002) New heart health claim for soy protein, *Food Aust.* **54**, 490.
- Gebara OC, Mittleman MA, Sutherland P, Lipinska I, Matheney T, Xu P, Welty FK, Wilson PW, Levy D (1995) Association between increased estrogen status and increased fibrinolytic potential in the Framingham Offspring Study. *Circulation* **91**,1952–1958.
- Gennari C (1997) Ipriflavone: Background, *Calcif Tissue Int* **61**, S3-S4.
- Gilani GS & Anderson JJB (eds) (2002) Phytoestrogens and health. *AOCS Press*.

- Gilliland SE & Walker DK (1990) Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans *J Dairy Sci* **73**, 905 – 911.
- Goldwyn S, Lazinsky A & Wei H (2000) Promotion of health by soy isoflavones: efficacy, benefit and safety concerns. *Drug Metab Drug Interact* **17**, 261 - 289.
- Gomes AMP & Malcata FX (1999) *Bifidobacterium* spp. and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trends Food Sci Technol* **10**, 139 – 157.
- Goodman MT, Wilkens LR, Hankin JH, Lyu L-C, Wu AH, Kolonel LN (1997) Association of soy and fiber consumption with the risk of endometrial cancer. *Am J Epidemiol* **146**, 294–306.
- Grafton TF & Hansen DK (1998) In vitro embryotoxicity of genistein. *Teratology* **57**, 252.
- Graves AB, Larson EB, White LR, Teng EL & Homma A (1994) Cross-cultural research and perspectives: opportunities and challenges in international collaborative epidemiologic research of dementia and its subtypes: studies between Japan and the U.S. *Intern Psychoger* **6**, 209 - 223.
- Greendale GA, FitzGerald G, Huang MH, Sternfeld B, Gold E, Seeman T, Sherman S & Sowers M (2002) Dietary soy isoflavones and bone mineral density: Results from the Study of Women's Health across the Nation *Am J Epidemiol* **155**, 746-754.
- Greenwood S, Barnes S, ClarksonTB, Eden J, Helferich WG, Hughes C, Messina M & Setchell KDR (2000) The role of isoflavones in menopausal health: Consensus opinion of the North American Menopause Society, *Menopause* **7**, 215-229.
- Griffin BA (1999) Lipoprotein atherogenicity: an overview of current mechanisms *Proc Nutr Soc* **58**, 163-169.
- Grodstein F, Mayeux R & Stampfer MJ (2000) Tofu and cognitive function: food for thought. *J Am Coll Nutr* **19**, 207-209.

- Grun IU, Adhikari K, Li C, Li Y, Lin B, Zhang J & Fernando LN (2001) Changes in the profile of genistein, daidzein, and their conjugates during thermal processing of tofu. *J Agric Food Chem* **49**, 2839–2843.
- Hak AE, Polderman KH, Westendorp IC, Jakobs C, Hofman A, Witteman JC & Stehouwer CD (2000) Increased plasma homocysteine after menopause. *Atherosclerosis* **49**, 163–168.
- Hargreaves DF, Potten CS, Harding C, Shaw LE, Morton MS, Roberts SA, Howell A & Bundred NJ (1999) Two-week dietary soy supplementation has an estrogenic effect on normal premenopausal breast. *J Clin Endocrinol Metab* **84**, 4017-4024.
- Harrison RM, Phillippi PP, Swan KF & Henson MC (1999) Effect of genistein on steroid hormone production in the pregnant rhesus monkey. *Exp Biol Med* **222**, 78-84.
- Hawrylewicz EJ, Huang HH & Blair WH (1991) Dietary soybean isolate and methionine supplementation affect mammary tumour progression in rats. *J Nutr* **121**, 1693-1698.
- Hayes SA, Unklesbay N & Grun I (2001) *Isoflavone stability in chocolate beverages* [Abstract]. In: 222nd ACS Natl. Meeting; Chicago, Ill.; 26-30 Aug 2001. Washington, D.C.: American Chemical Society.
- Heinonen S, Wahala K & Adlercreutz H (2000) Metabolites of dietary phytoestrogens daidzein, genistein, and glycitein. *J Nutr* **130**, 683S.
- Hendrich S & Murphy PA (2001) Isoflavones: Source and metabolism. In Wildman REC, editor. *Handbook of Nutraceuticals and Functional Foods*. Boca Raton, Fla.: CRC Press. Pp. 55-75.
- Hendrich S, Wang GJ, & Lin HK (1999) *Isoflavone metabolism and bioavailability*. In: Papas AM, ed. *Antioxidant Status, Diet, Nutrition and Health*. Boca Raton, FL: CRC Press p211-230.
- Hilakivi-Clarke L, Onojafe I & Raygada M (1999) Prepubertal exposure to zearalenone or genistein reduces mammary tumorigenesis. *Br J Cancer* **80**, 1682-1688.

- Hodgson JM, Puddey IB, Beilin LJ, Mori TA & Croft KD (1998) Supplementation with isoflavonoid phytoestrogens does not alter serum lipid concentrations - a randomized controlled trial in humans *J Nutr* **128**, 728-732.
- Hodgson JM, Puddey IB, Croft KD, Mori TA, Rivera J & Beilin LJ (1999) Isoflavonoids do not inhibit in vivo lipid peroxidation in subjects with high-normal blood pressure *Atherosclerosis* **145**, 167-172.
- Honore EK, Williams JK, Anthony MS & Clarkson TB (1997) Soy isoflavones enhance coronary vascular reactivity in atherosclerotic female macaques. *Fertil Steril* **67**, 148–54.
- Horn-Ross PL, John EM, Lee M, Stewart SL, Koo J, Sakoda LC, Shiau AG, Goldstein J, Davis P & Perez-Stable EJ (2001) Phytoestrogen consumption and breast cancer risk in a multiethnic population - The Bay Area Breast Cancer Study, *Am J Epidemiol* **154**, 434-441.
- Howes JB, Sullivan D, Lai N, Nestel P, Pomeroy S, West L, Eden J A & Howes LG (2000) The effects of dietary supplementation with isoflavones from red clover on the lipoprotein profiles of post menopausal women with mild to moderate hypercholesterolaemia, *Atherosclerosis* **152**, 143-147.
- Hsu CA, Yu RC & Chou CC (2005) Production of β -galactosidase by Bifidobacteria as influenced by various culture conditions. *Int J Food Microbiol* **104**, 197 – 206.
- Huggett AC, Pridmore S, Malnoe A, Haschke F, Offord EA (1997) Phyto-oestrogens in soy-based infant formula. *Lancet* **350**, 815–816.
- Husband AJ (2002) Phytoestrogens and menopause - Published evidence supports a role for phytoestrogens in menopause *Br Med J* **324**, 52.
- Hutchins AM, Slavin JL & Lampe JW (1995) Urinary isoflavonoid phytoestrogen and lignan excretion after consumption of fermented and unfermented soy products. *J Am Diet Assoc* **95**, 545 - 551.

- Hutt P, Shchepetova J, Loivukene K, Kullisaar T & Mikelsaar M (2006) Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. *J Appl Microbiol* **100**, 1324 – 1332.
- Inchaurredo VA, Flores MV & Voget CE (1998) Growth and β -galactosidase synthesis in aerobic chemostat cultures of *Kluyveromyces lactis*. *J Ind Microbiol Biotech* **20**, 291 – 298.
- Ingram D, Sanders K, Kolybaba M & Lopez D (1997) Case-control study of phyto-oestrogens and breast cancer *Lancet* **350**, 990-994.
- Ingram D, Sanders K, Kolybaba M & Lopez D (1997) Casecontrol study of phyto-oestrogens and breast cancer. *Lancet* **350**, 990-994.
- Ismail B & Hayes K (2005) β -Glucosidase activity toward different glycosidic forms of isoflavones. *J Agric Food Chem* **53**, 4918 - 4924.
- Isolauri E, Sütas Y, Kankaanpää P, Arvilommi H & Salmien S (2001) Probiotics: effects on immunity. *Am J Clin Nutr* **73**, 444S – 450S.
- Izumi T, Piskula MK, 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. *J Nutr* **130**, 1695 - 1699.
- Jakes RW, Duffy SW, Ng FC, Gao F, Ng EH, Seow A, Lee HP & Yu MC (2002) Mammographic parenchymal patterns and self-reported soy intake in Singapore Chinese women *Cancer Epidemiol Biomarkers Prev* **11**, 608-613.
- Jenkins DJA, Kendall CWC, Garsetti M, Rosenberg-Zand RS, Jackson CJ, Agarwal S, Rao AV, Diamandis EP, Parker T, Faulkner D, VuksanV & Vidgen E (2000) Effect of soy protein foods on low-density lipoprotein oxidation and ex vivo sex hormone receptor activity - A controlled crossover trial *Metabolism* **49**, 537-543.
- Jenkins DJA, Kendall CWC, Jackson CC, Connelly PW, Parker T, Faulkner D, Vidgen E, Cunnane SC, Leiter LA & Josse RG (2002) Effects of high and low isoflavones soyfoods on

- blood lipids, oxidised LDL, homocysteine, and blood pressure in hyperlipidemic men and women. *Am J Clin Nutr* **76**, 365 – 372.
- Joannou GE, Kelly GE, Reeder AY, Waring M & Nelson C (1995) A urinary profile of dietary phytoestrogens. The identification and mode of metabolism of new isoflavonoids. *J Steroid Biochem Mol Biol* **54**, 167 – 184.
- Jorgensen K, Suter DAI, Thompson WK, Dalais FS, Wahlqvist ML & Rice GE (1999) Burgen soy-lin: Development of an innovative functional staple food, *Functional Foods* **2**, 22-24.
- Kandler O & Weiss N. (1986). Genus *Lactobacillus*, In P. H. A. Sneath, N. S. Mair, and M. E. Sharp (ed.), *Bergey's manual of systematic bacteriology*, vol. 2. The Williams & Wilkins Co., Baltimore, Md. p. 1209–1234.
- Kapiotis S, Hermann M, Held I, Seelos C, Ehringer H & Gmeiner BMK (1997) Genistein, the dietary-derived angiogenesis inhibitor, prevents LDL oxidation and protects endothelial cells from damage by atherogenic LDL *Atheroscler Thromb Vasc Biol* **17**, 2868-2874.
- Kelsey JL & Horn-Ross PL (1993) Breast cancer: magnitude of the problem and description epidemiology. *Epidemiol Rev* **15**, 7-16.
- Kerry N & Abbey M (1998) The isoflavone genistein inhibits copper and peroxy radical mediated low density lipoprotein oxidation in vitro *Atherosclerosis* **140**, 341-347.
- Keusch P (2005) Chemical kinetics: Rate laws, Arrhenius equation and experiments. University of Regensburg, Germany. www.uni-regensburg.de (19/04/2005)
- Kim H, Xia H, Li L & Gewin J (2000) Attenuation of neurodegeneration-relevant modifications of brain proteins by dietary soy. *Biofactors* **12**, 243-250.
- King RA & Bignell CM (2000) Concentrations of isoflavone phytoestrogens and their glucosides in Australian soya beans and soya foods. *Aust J Nutr Diet* **57**, 2, 70 -78.
- King RA (1998) Daidzein conjugates are more bioavailable than genistein conjugates in rats. *Am J Clin Nutr* **68**, 1496S–1499S.

- King RA (2002) Digestion, absorption and metabolism of isoflavones. In: Phytoestrogens and health, Editors: G.S.Gilani and J.J.B.Anderson. AOCS Press.
- King RA, Broadbent JL, & Head RJ (1996) Absorption and excretion of the soy isoflavone genistein in rats. *J Nutr* **126**, 176-182
- Knight DC & Eden JA (1994) A review of the clinical effects of phytoestrogens. *Obstet Gynecol* **87**, 897- 904.
- Knight DC & Eden JA (1995) Phytoestrogens: a short review. *Maturitas* **22**,167 - 175.
- Knight DC, Eden J A, Huang JL & Waring MA (1998) Isoflavone content of infant foods and formulas. *J Paed Child Health* **34**, 135 – 138.
- Komatsu Y, Suematsu S, Hisanobu Y, Saigo H, Matsuda R & Hara K (1993) Effects of pH and temperature on reaction kinetics of catechins in green tea infusion. *Biosci Biotech Biochem* **57**, 907 – 910.
- Krzewinski F, Brassart C, Gavini F & Bouquelet S (1996) Characterisation of the lactose transport system in the strain *Bifidobacterium bifidum* DSM 20082. *Current Microbiol* **32**, 301 - 307
- Kudou S, Fleury Y, Welti D, Magnolato D, Uchida T, Kitamura K & Okubo K (1991) Malonyl isoflavone glycosides in soybean seeds (*Glycine max* Merrill). *Agric Biol Chem* **55**, 2227–2233.
- Kulling SE, Honig DM & Metzler M (2000) Oxidative in vitro metabolism of the soy phytoestrogens daidzein and genistein. *J Agric Food Chem* **48**, 4963 - 4972.
- Kurzer MS (2000) Hormonal effects of soy isoflavones: Studies in premenopausal and postmenopausal women. *J Nutr* **130**, 660S.
- Kyle E, Neckers L, Takimoto C, Curt G & Bergan R (1997) Genistein-induced apoptosis of prostate cancer cells is preceded by a specific decrease in focal adhesion kinase activity. *Mol Pharmacol* **51**, 193-200.

- Labuza TP (2000) Functional foods and dietary supplements: product safety, good manufacturing practice regulations, and stability testing. In: Schmidl MK, Labuza TP, editors. Essentials of functional foods. Gaithersburg, Md.: Aspen Publishers Inc. p 15–48.
- Lamartiniere CA (2000) Protection against breast cancer with genistein: a component of soy. *Am J Clin Nutr* **71**, 1705S-1709S.
- Lamartiniere CA, Murrill WB, Manzollillo PA, Zhang JX, Barnes S, Zhang XS, Wei HC & Brown NM (1998) Genistein alters the ontogeny of mammary gland development and protects against chemically-induced mammary cancer in rats, *Proc Soc Exp Biol Med* **217**, 358-364.
- Lamartiniere CA, Zhang J-X & Cotroneo MS (1998) Genistein studies in rats: potential for breast cancer prevention and reproductive and developmental toxicity. *Am J Clin Nutr* **68**, 1400S-1405S.
- Lee HP, Gourley L, Duffy SW, Esteve J, Lee J, Day NE (1992) Risk factors for breast cancer by age and menopausal status: a case-control study in Singapore. *Cancer Causes Control* **3**, 313–322.
- Lee HP, Gourley L, Duffy SW, Esteve J, Lee J & Day NE (1992) Risk factors for breast cancer by age and menopausal status: a case-control study in Singapore. *Cancer Causes Control* **3**, 313-322.
- Lee SJ, Ahn JK, Kim SH, Kim JT, Han SJ, Jung MY & Chung IM (2003) Variation in isoflavones of soybean cultivars with location and storage duration. *J Agric Food Chem* **51**, 3382–3389.
- Lees CJ & Ginn TA (1998) Soy protein isolate diet does not prevent increased cortical bone turnover in ovariectomized macaques *Calcif.Tissue Int* **62**, 557-558.
- Levy JR, Faber KA, Ayyash L & Hughes CL Jr (1995) The effect of prenatal exposure to the phytoestrogen genistein on sexual differentiation in rats. *Proc Soc Exp Biol Med* **208**, 60-66.

- Liu K (1997) *Soybeans: Chemistry, Technology, and Utilization*. New York: Chapman and Hall. p 415 – 418.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. *J Agric Food Chem* **47**, 2697–2704.
- Lu L-JW & Anderson KE (1998) Sex and long-term soy diets affect the metabolism and excretion of soy isoflavones in humans. *Am J Clin Nutr* **68**, 1500S–1504S.
- Lu L-JW, Cree M, Josyula S, Nagamani M, Grady JJ & Anderson KE (2000) Increased urinary excretion of 2-hydroxyestrone but not 16 alpha-hydroxyestrone in premenopausal women during a soya diet containing isoflavones. *Cancer Res* **60**, 1299-1305.
- Mahungu SM, Diaz-Mercado S, Li J, Schwenk M, Singeltary K & Faller J (1999) Stability of isoflavones during extrusion processing of corn/soy mixture. *J Agric Food Chem* **47**, 279–284.
- Makela S, Savolainen H, Aavik E, Myllarniemi M, Strauss L, Taskinen E, Gustafsson JA & Hayry P (1999) Differentiation between vasculoprotective and uterotrophic effects of ligands with different binding affinities to estrogen receptors alpha and beta. *Proc Natl Acad Sci USA* **96**, 7077–82.
- Maskarinec G, Singh S, Meng L, Franke AA (1998) Dietary soy intake and urinary isoflavone excretion among women from a multiethnic population. *Cancer Epidemiol Biomarkers Prev* **7**, 613–619.
- Matthews KA, Kuller LH, Sutton-Tyrrell K & Chang YF (2001) Changes in cardiovascular risk factors during the perimenopause and postmenopause and carotid artery atherosclerosis in healthy women. *Stroke* **32**, 1104–11.
- Maugeri D, Panebianco P, Russo MS, Motta M, Tropea S, Motta L, et al. (1994) Ipriflavone treatment of senile osteoporosis: results of a multicenter, double-blind clinical trial of 2 years. *Arch Gerontol Geriatr* **19**, 253–263.

- Maugeri D, Panebianco P, Russo MS, Motta M, Tropea S, Motta L, Garozzo C, Lomeo E, Barbagallo Sangiorgi G, Scuderi G, Carozzo M, Cantatore FP, Perpignano G, Ferraraccio A & Ennas F (1994) Ipriflavone-treatment of senile osteoporosis: Results of a multicenter, double-blind clinical trial of 2 years *Arch Gerontol Geriatr* **19**, 253-263.
- McCormic L & Savage D (1983) Characterisation of *Lactobacillus sp.* strain 100-37 from the murine gastrointestinal tract; ecology, plasmid content and antagonistic activity toward *Clostridium*. *Appl Environ Microbiol* **46**, 1103-1112.
- McMichael-Phillips DF, Harding C & Morton M (1998) Effects of soy-protein supplementation on epithelial proliferation in histologically normal human breast. *Am J Clin Nutr* **68**, 1431 S-1436S.
- Mellentin J & Heasman M (2001) The land of soy milk and money *Dairy Ind Int* **66**, 11.
- Messina M, Gardner C & Barnes S (2002) Gaining insight into the health effects of soy but a long way still to go: Commentary on the Fourth International Symposium on the Role of Soy in Preventing and Treating Chronic Disease *J Nutr* **132**, 547S-551S.
- Messina MJ & Loprinzi CL (2001) Soy for breast cancer survivors: A critical review of the literature *J Nutr* **131**, 3095S-3108S.
- Metzger D, Berry M, Ali S & Chamber P (1995) Effect of antagonists on DNA binding properties of the human estrogen receptor in vitro and in vivo. *Mol Endocrinol* **9**, 579-591.
- Miksicek RJ (1995) Estrogenic flavonoids: Structural requirements for biological activity. *Proc Soc Exp Biol Med*, **208**, 44-50.
- Mitsuoka T (1990) Role of intestinal flora in health with special reference to dietary control of intestinal flora. In: *Microbiology Applications in Food Biotechnology* B. H. Nga and Y.K. Lee, Eds.). Elsevier, Essex. 135p
- Morabito N, Crisafulli A, Vergara C, Gaudio A, Lasco A, Frisina N, Danna R, Corrado F, Pizzoleo M A, Cincotta M, Altavilla D, Ientile R & Squadrito F (2002) Effects of genistein

- and hormone-replacement therapy on bone loss in early postmenopausal women: A randomized double-blind placebo-controlled study *J Bone Min Res* **17**, 1904-1912.
- Morton MS, Chan PSF, Cheng C, et al (1997) Lignans and isoflavonoids in plasma and prostatic fluid in men: samples from Portugal, Hong Kong, and the United Kingdom. *Prostate* **32**, 122–128.
- Mosca L, Manson JE, Sutherland SE, Langer RD, Manolio T & Barrett- Connor E (1997) Cardiovascular disease in women: a statement for healthcare professionals Professionals from the American Heart Association. Writing Group. *Circulation* **96**, 2468–82.
- Munro IC, Harwood M, Hlywka JJ, Stephen AM, Doull J & Flamm WG (2003) Soy isoflavones: A safety review. *Nutr Rev* **61**, 1-33.
- Murkies AL, Lombard C, Strauss BJG, Wilcox G, Burger HG & Morton MS (1995) Dietary flour supplementation decreases postmenopausal hot flushes: effect of soy and wheat. *Maturitas* **21**, 189-195.
- Murphy P, Song T, Buseman G, Barua K, Beecher GR, Trainer D & Holden J (1999) Isoflavone in retail and institutional soy foods. *J Agric Food Chem*, **47**, 2697-2704.
- Nagata C, Shimizu H, Takami R, Hayashi M, Takeda N & Yasuda K (2002) Soy product intake and serum isoflavonoid and estradiol concentrations in relation to bone mineral density in postmenopausal Japanese women *Osteoporos Int* **13**, 200-204.
- Nagata C, Takatsuka N, Kurisu Y, Shimizu H (1998) Decreased serum total cholesterol concentration is associated with high intake of soy products in Japanese men and women. *J. Nutr* **128**, 209 - 213.
- Nagy Z, Keresztessy Z, Szentirmai A & Biro S (2001) Carbon source regulation of β -galactosidase biosynthesis in *Penicillium chrysogenum*: production, purification, and characterisation of the enzyme. *Protein Expr Purif* **21**, 24 – 29.
- National report (2001-2002) Consumer attitudes about nutrition 2002. United Soybean Board.

- National Research Council, Subcommittee on Swine Nutrition, Committee on Animal Nutrition Board on Agriculture. (1988) Nutrient requirements of swine, 10th revised ed. Washington, DC: National Academy Press.
- Nestel P (2002) Role of soy protein in cholesterol-lowering - How good is it?, *Atheroscler Thromb Vasc Biol* **22**, 1743-1744.
- Nomura A, Henderson BE & Lee J (1978) Breast cancer and diet among the Japanese in Hawaii. *Am J Clin Nutr* **31**, 2020-2025.
- O'Brien T & Nguyen TT (1997) Lipids and lipoproteins in women. *Mayo Clin Proc* **72**, 235-244.
- Ogawa T, Hirai R, Nakakuni H, Sato Y, Wakisaka S, Tachibana M, Tominaga H, Kurata M & Matsubayashi K (1974) Clinical experience with the use of high concentration lactic acid bacteria preparation LP-201 to treat constipation. *Clin Rep* **6**, 1085-1092.
- Ohta A, Uehara M, Sakai K, Takasaki M, Adlercreutz H, Morohashi T & Ishimi Y (2002) A combination of dietary fructooligosaccharides and isoflavone conjugates increases femoral bone mineral density and equol production in ovariectomized mice *J Nutr* **132**, 2048-2054.
- Ollis WD (1962) *The isoflavonoids*. In: Geissman TA (editor). *The Chemistry of Flavonoid Compounds*. Newyork: Pergamon Press. 353 - 405p.
- Ono R & Yamaguchi M (1999) Increase in Bone Components of Rats Orally Administered Isoflavone-containing Soybean Extract (Nijiru) *J Health Sci* **45**, 66-69.
- Onozawa M, Kawamori T & Baba M (1999) Effects of a soybean isoflavone mixture on carcinogenesis in prostate and seminal vesicles of F344 rats. *Jpn J Cancer Res* **90**, 393-398.
- Orrhage K & Nord CE (2000) Bifidobacteria and lactobacilli in human health. *Drugs exp Clin Res* **26**, 95 -111.
- Ososki AL & Kennelly EJ (2003) Phytoestrogens: a review of the present state of research. *Phytotherapy Res* **17**, 845-868

- Pahk AH & DeLong MJ (1998) Vitamin D3 and genistein induce detoxification enzymes in MCF-7 human breast cancer cells [abstract]. *Toxicologist* **42**, 317.
- Park YK, Aguiar CL, Alencar SM, Mascrenhas HAA & Scamparini ARP (2002) Conversion of malonyl β -glucoside isoflavones into glycoside isoflavones found in some cultivars of Brazilian soybeans. *Ciênc Technol Aliment* **22**, 130-135.
- Park YK, Lui MCY & Aguiar CL (2003) Production of enriched isoflavone aglycones during processing of soy protein isolates and soy protein concentrates (Abstract). In: *2003 IFT Annual Meeting Book of Abstracts*; 12 – 16 July 2003; Chicago, IL. : Institute of Food Technologists, p 215.
- Peterson G & Barnes S (1993) Genistein and biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate* **22**, 335-345.
- Pierce, a Perbio Science Company: Technical resource 2003, Protein stability and storage. Rockford, IL, USA. (www.piercenet.com).
- Pino AM, Valladares LE, Palma MA, Mancilla AM, Yanez M & Albala C (2000) Dietary isoflavones affect sex hormone-binding globulin levels in postmenopausal women. *J Clin Endocrinol Metab* **85**, 2797-2800.
- Playne MJ (2002) The health benefits of probiotics. *Food Aust* **54**, 71 - 74.
- Postema MDH, Piper JL & Betts RL (2005) RCM-Based synthesis of a variety of β -C-glycosides and their in vitro anti-solid tumor activity. *J Org Chem* **70**, 829 – 836.
- Prasain JK, Jones K, Kirk M, Wilson L, Johnson MS, Weaver C & Barnes S (2003) Profiling and quantification of isoflavonoids in Kudzu dietary supplements by high- performance liquid chromatography and electrospray ionization tandem mass spectrometry. *J Agric Food Chem* **51**, 4213 – 4218.
- Prasain JK, Ueki M, Stefanowicz P & Osada H (2002) Rapid screening and identification of cytochalacins by electrospray tandem mass spectrometry. *J Mass Spec* **37**, 283 – 291.

Pszczola DE (2000) Soy: Why it's moving into the mainstream *Food Tech* **54**, 76-85.

Quella SK, Loprinzi CL, Barton DL, Knost JA, Sloan JA, LaVasseur BI, Swan D, Krupp KR.,

Miller KD & Novotny PJ. (2000) Evaluation of soy phytoestrogens for the treatment of hot flashes in breast cancer survivors: A North Central Cancer Treatment Group Trial. *J Clin Oncol* **18**, 5, 1068-1074.

Rackis JJ (1979) Evaluation of the Health Aspects of Soy Protein Isolates as Food Ingredients,

Prepared for FDA by Life Sciences Research Office, *Federation of American Societies for Experimental Biology*, 9650 Rockville Pike, Bethesda, MD 20014, Contract No, FDA 223-75-2004.

Rajala S, Salminen S, Seppanen J & Vapaatalo H (1988) Treatment of chronic constipation with

lactitol sweetened yogurt supplemented with guar gum and wheat bran in elderly hospital patients. *Comp Gerontol* **2**, 83-86.

Reimann JD, Anja C, Duncan T, Marika F, Jesper H, Wendy LH, Katerina V, Ulrich H,

Francesco B, Susanne B, Christine M W, Zunft HJF & Corinna K (2006) Consumption of Soy Isoflavones Does Not Affect Plasma Total Homocysteine or Asymmetric Dimethylarginine Concentrations in Healthy Postmenopausal Women *J Nutr* **136**, 100–105.

Reinli K & Block G (1996) Phytoestrogen content of foods: a compendium of literature values.

Nutr Cancer **26**, 123 - 148.

Riaz MN (2006) Soy applications in food. Boca Raton, Fla.; London; CRC/Taylor & Francis. P1

- 13

Rice MM, Graves AB, McCurry SM (1999) Tofu consumption and cognition in older Japanese

American men and women. In: Messian M, ed. Third International Symposium on the Role of Soy in Preventing and Treating Chronic Disease Program. October 31-November 3, 1999. Washington, DC: United Soybean Board/American Soybean Association p27.

Richardson T, Hyslop DB (1985) *Enzymes*. In: Owen R. Fennema (editor). Food Chemistry 2nd

Edition and Expanded. New York: Marcel Dekker. p 400 – 403.

- Richelle M, Pridmore-Merten S, Bodenstab S, Enslin M & Offord EA (2002) Hydrolysis of isoflavone glycosides to aglycones by β -glucosidase does not alter plasma and urine isoflavone pharmacokinetics in post menopausal women. *J Nutr* **132**, 2587 – 2592.
- Rosenberge IM (1996) Protein Analysis and Purification: Benchtop Techniques. Boston: Birkhauser Publishing. 105 – 107p.
- Samman S, Wall PML, Chan GSM, Smith SJ & Petocz P (1999) The effect of supplementation with isoflavones on plasma lipids and oxidisability of low density lipoprotein in premenopausal women. *Atherosclerosis* **147**, 277-283.
- Sanders ME, Walker DC, Walker KM, Aoyama K & Klaenhammer TR (1996) Performance of commercial cultures in fluid milk applications. *J Dairy Sci* **79**, 943– 955.
- Sathyamoorthy N, Wang TTY, Phang JM (1994) Stimulation of pS2 expression by diet derived compounds. *Cancer Res* **54** 957-961.
- Scalabrini P, Rossi M, Spettoli P & Matteuzi D (1998) Characterisation of *Bifidobacterium* stains for use in soymilk fermentation. *Intern J Food Microbiol* **39**, 213–219
- Scambia G, Mango D & Signorite PG (2000) Clinical effects of a standardized soy extract in postmenopausal women: a pilot study. *Menopause* **7**, 105-111.
- Scardovi V & Trovattelli LD (1965) The fructose-6-phosphate shunt as peculiar pattern of hexose degradation in the genus *Bifidobacterium*. *Ann Microbiol* **15**, 19 – 29.
- Scheiber MD, Liu JH, Subbiah MTR, Rebar RW & Setchell KDR (2001) Dietary inclusion of whole soy foods results in significant reductions in clinical risk factors for osteoporosis and cardiovascular disease in normal postmenopausal women. *Menopause* **8**, 384-392.
- Setchell KDR & Adlercreutz H (1988) *Mammalian lignans and phyto-oestrogens: recent studies on their formation, metabolism and biological role in health and disease*. In: Rowland IR, ed. Role of the Gut Flora in Toxicity and Cancer. New York: Academic Press Ltd, p315 -345.
- Setchell KDR & Cassidy A (1999) Dietary isoflavones: Biological effects and relevance to human health. *J Nutr* **129**, S758 - S767.

- Setchell KDR (2000) Absorption and metabolism of soy isoflavones-from food to dietary supplements and adults to infants. *J Nutr* **130**, 654S - 655S.
- Setchell KDR, Borriello SP, Hulme P, Kirk DN & Axelson M (1981) Non steroidal estrogens of dietary origin; possible roles in relation to cancer. *Lancet* **2**, 4 – 8.
- Setchell KDR, Brown MN, Zimmer-Nechemias L, Wolfe BE, Brashear WT, Kirschner A, Cassidy A & Heubi JE (2001) Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J Nutr* **131**, 1362S -1375S.
- Setchell KDR, Brown MN, Zimmer-Nechemias L, Wolfe BE, Brashear WT, Kirschner A, Cassidy A & Heubi JE (2002b) Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am J Clin Nutr* **76**, 447-453.
- Setchell KDR, Brown NM & Lydeking-Olsen E (2002c) The clinical importance of the metabolite equol- A clue to the effectiveness of soy an dits isoflavones. *J Nutr* **132**, 3577 – 3584.
- Setchell KDR, Gosselin S J, Welsh MB, Johnston JO, Balistreri WF, Kramer LW, Dresser BL & Tarr MJ (1987a) Dietary estrogens; a probable cause of infertility and liver disease in captive cheetahs. *Gastroenterology* **93**, 225 – 233.
- Setchell KDR, Maynard-Brown N, Desai PB, Zimmer-Nechemias L, Wolfe B, Jakate AS, Creutzinger V & Heubi JE (2003) Bioavailability, disposition, and dose-response effects of soy isoflavones when consumed by healthy women at physiologically typical dietary intakes. *J Nutr* **133**, 1027-1035.
- Setchell KDR, Welsh MB & Lim CK (1987b) HPLC analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical, and thermospray, mass spectrometric detection. *J. Chromatogr* **386**, 315-323.

- Shah NP & Lankaputhra WEV (2002) *Bifidobacterium* spp: morphology and physiology. In *Encyclopedia of Dairy Sciences*, pp. 141 – 146, [H Roginski, JW Fuquay & PF Fox, editors]. London: Academic Press.
- Shah NP (2000) Probiotic bacteria: Selective enumeration and survival in dairy foods. *J Dairy Sci* **83**, 894 – 907.
- Shaikh SA, Khire JM & Khan MI (1997) Production of β -galactosidase from thermophilic fungus *Rhizomucor* sp. *J Ind Microbiol Biotech* **19**, 239 – 245.
- Sharpe ME (1981) The genus *Lactobacillus*, In M. P. Starr, H. Stolp, H. G. Truper, A. Ballows, and H. G. Schlegel (ed.), *The procaryotes*. Springer-Verlag, Berlin. p. 1653-1674.
- Shimoni E (2004) Stability and shelf life of bioactive compounds during food processing and storage: Soy isoflavones. *J Food Sci* **69**, 6, 160-166.
- Singletary K, Faller J, Li JY & Mahungu S (2000) Effect of extrusion on isoflavone content and antiproliferative bioactivity of soy/corn mixtures. *J Agric Food Chem* **48**, 3566–3571.
- Slavin JL, Karr SC, Hutchins AM & Lempe JW (1998) Influence of soybean processing, habitual diet, and soy dose on urinary isoflavonoid excretion. *Am J Clin Nutr* **68**, 1492S – 1495S.
- Slikker W Jr, Scallet AC, Doerge DR & Ferguson SA (2001) Gender-based differences in rats after chronic dietary exposure to genistein. *Int J Toxicol* **20**, 175-179.
- Slominski BA (1994) Hydrolysis of galactooligosaccharides by commercial preparations of α -galactosidase and β -fructofuranose: Potential for use as dietary additives. *J Sci Food Agric* **65**, 323-330.
- Song TT, Hendrich S & Murphy PA (1999) Estrogenic activity of glycitein, a soy isoflavone. *J Agric Food Chem* **47**, 1607-1610.
- Spence K (2000) National Food's new soy beverage plant *Food Aust* **52**, 303.
- St-Onge M-P, Fanworth ER & Jones PJH (2000) Consumption of fermented and non-fermented dairy products: effects on cholesterol concentrations and metabolism. *Am J Clin Nutr* **71**, 674 – 681.

- Strauss L, Makela S, Joshi S, Huhtaniemi I & Santti R (1998) Genistein exerts estrogen-like effects in male mouse reproductive tract. *Mol Cell Endocrinol* **144**, 83-93.
- Strom, S. S., Yamamura, Y., Duphorne, C. M., Splitz, M. R., Babaian, R. J., & Pillow, P. C. (1999). Phytoestrogen intake and prostate cancer, a case control study using new database. *Nutrition Cancer* **33**, 20–25.
- Tannock GW (1999) *Probiotics: a critical review*. Norfolk, England: Horizon Scientific Press.
- Teede H J, Dalais FS, Kotsopoulos D, Liang YL, Davis S & Mcgrath BP (2001) Dietary soy has both beneficial and potentially adverse cardiovascular effects: A placebo-controlled study in men and postmenopausal women *J Clin Endocrinol Metab* **86**, 3053-3060.
- Tharmaraj N & Shah NP (2003) Selective enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, Bifidobacteria, *Lactobacillus casei*, *Lactobacillus rhamnosus* and Propionibacteria. *J Dairy Sci* **86**, 2288-2296.
- Tissier H (1899). Le Bacterium coli et le rhction chromophilr d' Bcherich. C.R. *Soc Biol* **51**, 943. (In French)
- Tochikura T, Sakai K, Fujiyoshi T, Tachiki T & Kumagai H (1986) p-Nitrophenyl glycoside-hydrolyzing activities in bifidobacteria and characterisation of β-D-galactosidase of *Bifidobacterium longum* 401. *Agric Biol Chem* **50**, 9, 2279 – 2286.
- Tsangalis D & Shah N P (2004). Metabolism of oligosaccharides and aldehydes and production of organic acids in soymilk by probiotic bifidobacteria. *Int J Food Sci Tech* **39**, 541 – 554.
- Tsangalis D (2004) Composite effects of isoflavones and Bifidobacteria on lipid profiles, bone turnover and sex steroid hormones in postmenopausal women. In Chapter 9.0, PhD thesis, Victoria University, Australia.
- Tsangalis D, Ashton J F, McGill AE J & Shah NP (2002) Enzymic transformation of isoflavone phytoestrogens in soymilk by β-glucosidase producing bifidobacteria. *J Food Sci* **67**, 8, 3104 - 3113.

- Tsangalis D, Ashton J F, McGill AE J & Shah NP (2003) Biotransformation of isoflavones by bifidobacteria in fermented soymilk supplemented with D-glucose and L-cysteine. *J Food Sci* **68**, 623 – 631
- Tsangalis D, Ashton JF, Stojanovska L, Wilcox G & Shah NP (2004) Development of an isoflavone aglycone enriched soymilk using soy germ, soy protein isolate and bifidobacteria. *Food Res Inter* **37**, 301-312.
- Tsukamoto C, Shimada S, Igita K, Kudou S, Kokubun M & Okubo K (1995) Factors affecting isoflavone content in soybean seeds – changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. *J Agric Food Chem* **43**, 1184-1192.
- Tsuruta O, Yuasa H, Kurono S, & Hashimoto H (1999) Syntheses of two trimannose analogs each containing C-mannosyl or 5-thio-C-mannosyl residue: their affinities to concanavalin A. *Bioorg Med Chem* **9**, 807-810.
- Uesugi T, Fukui Y & Yamori Y (2002) Beneficial effects of soybean isoflavone supplementation on bone metabolism and serum lipids in postmenopausal Japanese women: A four-week study *J Am Coll Nutr* **21**, 97-102.
- Uesugi T, Toda T, Tsuji K & Ishida H (2001) Comparative study on reduction of bone loss and lipid metabolism abnormality in ovariectomized rats by soy isoflavones, daidzin, genistin, and glycitin *Biol Pharm Bull* **24**, 368-372.
- Ungar Y, Osundahunsi OF & Shimoni E (2003) Thermal stability of genistein and daidzein and its effect on their antioxidant activity. *J Agric Food Chem* **51**, 2232-2236.
- Upmalis DH, Lobo R, Bradley L, Warren M, Cone FL & Lamia CA (2000) Vasomotor symptom relief by soy isoflavone extract tablets in postmenopausal women: A multicenter, double-blind, randomized, placebo-controlled study *Menopause* **7**, 236-242.

- Valachovicova T, Slivova V, Bergman H, Shuherk J & Sliva D (2004) Soy isoflavones suppress invasiveness of breast cancer cells by the inhibition of NF- κ B/AP-1-dependent and independent pathways. *Intern J Oncol* **25**, 1389 -1395.
- van der Schouw YT, Pijpe A, Lebrun CEI, Bots ML, Peeters PHM, van Staveren WA, Lamberts SWJ & Grobbee DE (2002) Higher usual dietary intake of phytoestrogens is associated with lower aortic stiffness in postmenopausal women *Atheroscler Thromb Vasc Biol* **22**, 1316-1322.
- Wakai K, Egami I & Kato K (1999) Dietary intake and sources of isoflavones among Japanese. *Nutr Cancer* **33**, 139-145.
- Walker HA, Dean TS, Sanders TAB, Jackson G, Ritter JM & Chowienczyk PJ (2001) The phytoestrogen genistein produces acute nitric oxide-dependent dilation of human forearm vasculature with similar potency to 17 beta-estradiol *Circulation* **103**, 258-262.
- Walter ED (1941) Isoflavone: a saponin glucoside in soya. *Justus Liebigs Ann. Chem.* **489**, 55 (in German).
- Wang C & Kurzer MS (1997) Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. *Nutr Cancer* **28**, 236-247.
- Wang C, Ma Q, Pagadala S, Sherrard MS & Kirshnan PG (1998) Changes of isoflavones during processing of soy protein isolates. *J Am Oil Chem Soc* **75**, 337–341.
- Wang CC, Prasain JK, & Barnes S (2002) Review of the methods used in the determination of phytoestrogens. *J Chromatogr B* **777**, 3 – 28.
- Wang G, Kuan SS, Francis OJ, Ware GM & Carman AS (1990) A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products. *J Agric Food Chem* **38**, 185–90.
- Wang H & Murphy PA (1994a) Isoflavone content in commercial foods. *J Agric Food Chem* **42**, 1666 – 1673.

- Wang H & Murphy PA (1994b) Isoflavone composition of American and Japanese soybeans in Iowa; Effects of variety, crop year and location. *J Agric Food Chem* **42**, 1674 – 1677.
- Wang HJ & Murphy PA (1996) Mass balance study of isoflavones during soybean processing. *J Agric Food Chem* **44**, 2377–83.
- Wang J, Kovac P, Sinay P & Glaudemans CPJ (1998) Synthetic C-oligosaccharides mimic their natural, analogous immunodeterminants in binding to three monoclonal immunoglobulins. *Carb Res* **308**, 191 – 193.
- Wang TTY, Sathyamoorthy N, Phang JM (1996) Molecular effects of genistein on estrogen receptor mediated pathways. *Carcinogenesis* **17**, 271-275.
- Waridel P, Wolfender JL, Ndjoko K, Hobby KR, & Major HJ (2001) Hostettmann, K. Evaluation of quadrupole time-of-flight tandem mass spectrometry for the differentiation of C-glycosidic flavonoid isomers. *J Chromatogr A*, **926**, 29 – 41.
- Watson WH, Cai J, & Jones DP (2000) Diet and apoptosis. *Ann Rev Nutr* **20**, 485 – 505.
- Wei A, Boy K, & Kishi YJ (1995) Biological evaluation of rationally modified analogs of the H-type-II blood-group trisaccharide - a correlation between solution conformation and binding-affinity. *J Amer Chem Soc* **117**, 9432 – 9436.
- Welch GN & Loscalzo J (1998) Homocysteine and atherothrombosis. *N Engl J Med* **338**, 1042–50.
- White L, Petrovitch H, Ross GW & Masaki K (1996) Association of mid-life consumption of tofu with late life cognitive impairment and dementia: The Honolulu Asia aging study. *Neurobiol Aging* **17**, 121S.
- White LR, Petrovitch H & Ross GW (2000) Brain aging and midlife tofu consumption. *J Am Coll Nutr* **19**, 242-255.
- Wingrove AS & Caret RL (1981) *Reaction Mechanisms*. In. Organic Chemistry. New York. Harper and Row, p148 - 157.

- Wiseman H (1997) Dietary phytoestrogens: disease prevention versus potential hazards. *Nutr Food Sci* **1**, 32-38.
- Wiseman H, O'Reilly JD, Adlercreutz H, Mallet AI, Bowey EA, Rowland IR & Sanders TAB (2000) Isoflavone phytoestrogens consumed in soy decrease F-2-isoprostane concentrations and increase resistance of low-density lipoprotein to oxidation in humans *Am J Clin Nutr* **72**, 395 – 400.
- Witte JS, Ursin G, Siemiatycki J, Thompson WD, Paganini-Hill A & Haile RW (1997) Diet and premenopausal bilateral breast cancer - a case-control study, *Breast Cancer Res Treat* **42**, 243-251.
- Wu AH, Wan P, Hankin J, Tseng CC, Yu MC & Pike MC (2002) Adolescent and adult soy intake and risk of breast cancer in Asian-Americans *Carcinogenesis* **23**, 1491-1496.
- Wu AH, Ziegler RG & Horn-Ross PL (1996) Tofu and risk of breast cancer in Asian-Americans. *Cancer Epidemiol Biomarkers Prev* **5**, 901-906.
- www.lclabs.com (2005) (Accessed November 2005)
- Xu X, Duncan AM, Wanagen KE & Kurzer MS (2000) Soy consumption alters endogenous estrogen metabolism in postmenopausal women. *Cancer Epidemiol Biomarkers Prev* **9**, 781 - 786.
- Xu Z, Wu Q & Godber SJ (2002) Stabilities of daidzin, glycitin, genistin, and generation of derivatives during heating. *J Agric Food Chem* **50**, 7402–7406.
- Yamaguchi M, Gao YH & Ma ZJ (2000) Synergistic effect of genistein and zinc on bone components in the femoral-metaphyseal tissues of female rats. *J Bone Miner Metab* **18**, 77–83.
- Yang G, Franck RW, Bitman R, Samadder P & Arthur G (2001) Synthesis and growth inhibitory properties of glucosamine-derived glycerolipids. *Organic Letters* **3**, 197 – 200.

- Yildirim A, Tokgozoglu SL, Oduncu T, Oto A, Haznedaroglu I, Akinci D, Koksal G, Sade E, Kirazli S & Kes S (2001) Soy protein diet significantly improves endothelial function and lipid parameters *Clin Cardiol* **24**, 711-716.
- Yoshitake T, Kiyohara Y & Kato I (1995) Incidence and risk factors of vascular dementia and Alzheimer's disease in a defined elderly Japanese population: The Hisayhama Study. *Neurology* **45**, 1161-1168.
- You L, Casanova M & Bartolucci EJ (2002) Combined effects of dietary phytoestrogen and synthetic endocrine-active compound on reproductive development in Sprague-Dawley rats: genistein and methoxychlor. *Toxicol Sci* **66**, 91-104.
- Zava DT & Duwe G (1997) Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. *Nutr Cancer* **27**, 31-40.
- Zhu QY, Zhang A, Tsang D, Huang Y, Chen ZY (1997) Stability of green tea catechins. *J Agric Food Chem* **45**, 4624 – 4628.
- Zimeri J & Tong CH (1999) Degradation kinetics of (–)-epigallocatechin gallate as a function of pH and dissolved oxygen in a liquid model system. *J Food Sci* **64**, 753–758.
- Zubik L & Meydani M (2003) Bioavailability of soybean isoflavones from aglycone and glucoside forms in American women. *Am J Clin Nutr* **77**, 1459 – 1465.

Appendix

A. Method for Enzyme assay for β -Glucosidase in Soymilk using ρ -Nitrophenol as Substrate

- Dilute the 0.5% solution of ρ -nitrophenol to 1mg/mL by adding 1 ml of ρ -nitrophenol to 49 mL of 0.01 M sodium phosphate buffer made up in a 50 mL volumetric flask
- Prepare a standard concentration of 10 μ g/mL by adding 1mL of diluted ρ -nitrophenol solution to 99 mL of 0.7 Nacl made in a 100 mL volumetric flask.
- Prepare a standard concentration of 20 μ g/mL by adding 1mL of diluted ρ -nitrophenol solution (1mg/mL) 49 mL of 0.7 Nacl made in a 50 mL volumetric flask
- Prepare a standard concentration of 40 μ g/mL by adding 2mL of diluted ρ -nitrophenol solution (1mg/mL) to 48 mL of 0.7 Nacl made in a 50 mL volumetric flask
- Prepare a standard concentration of 50 μ g/mL by adding 5mL of diluted ρ -nitrophenol solution (1mg/mL) to 95 mL of 0.7 Nacl made in a 100 mL volumetric flask
- Prepare a standard concentration of 80 μ g/mL by adding 4mL of diluted ρ -nitrophenol solution (1mg/mL) to 46 mL of 0.7 Nacl made in a 50 mL volumetric flask
- Add (for control) 1000 μ L of unfermented soymilk in a clean dry test tube. Add 1000 μ L of ρ -nitrophenol solution (1mg/ml) into a clean dry test tube to act as a blank
- Fermented /unfermented soymilk – 1000 μ L in a clean dry test tube
- Add 1000 μ L of pNPG, incubate for 15 min at 37°C
- Add 500 μ L of cold NaCo₃
- Add 1000 μ L of Nacl for control (Buffer + sample control)
- Add 1000 μ L of Nacl for blank (Only the buffer)

- Take absorbance readings using spectrophotometer at 420nm setting blank as a reference point.
- Draw a linear curve using regression analysis by plotting absorbance of p-nitrophenol verses the concentration

Assay for β -glucosidase activity in soymilk

- Take 10 mL of aliquot (fermented soymilk) in a sterilised bottle
- Add 1000 μ L of 5mM (0.005M) p-Nitrophenyl B-D-glucopyranoside to the aliquot and incubate at 37°C for 15 minutes
- Stop the reaction by adding 500 μ L cold 1 M of Sodium carbonate (Na_2CO_3)
- Measure the absorbance readings at 400nm using a spectrophotometer.
- Convert readings into enzyme activity, which is the amount of p-Nitrophenol released in μ mole per min at 37°C

Preparation of a blank

- Take 10 mL of aliquot (unfermented soymilk) in a sterilised bottle
- Add 500 μ L of 5mM p-Nitrophenyl B-D-glucopyranoside and 500 μ L of O-Nitrophenyl B-D-galactopyranoside to the aliquot and incubate at 37°C for 15 minutes
- Stop the reaction by adding 500 μ L cold 1 M of Sodium Carbonate (Na_2CO_3)
- Measure the absorbance readings at 420nm using a spectrophotometer.
- Set the obtained reading as reference (Zero/Blank)
- Begin to take sample reading after setting the reference using blank.

- Convert readings into enzyme activity, which is the amount of *p*-Nitrophenol released in μ mole per min at 37°C

B. Method for Enzyme assay for β -galactosidase in Soymilk using *O*-Nitrophenol as Substrate

- Dilute the 0.5% solution of *o*-nitrophenol to 1mg/mL by adding 1 ml of *o*-nitrophenol to 49 mL of 0.01 M sodium phosphate buffer made up in a 50 mL volumetric flask
- Prepare a standard concentration of 10 μ g/mL by adding 1mL of diluted *o*-nitrophenyl solution to 99 mL of 0.7 Nacl made in a 100 mL volumetric flask.
- Prepare a standard concentration of 20 μ g/mL by adding 1mL of diluted *o*-nitrophenyl solution (1mg/mL) 49 mL of 0.7 Nacl made in a 50 mL volumetric flask
- Prepare a standard concentration of 40 μ g/mL by adding 2mL of diluted *o*-nitrophenyl solution (1mg/mL) to 48 mL of 0.7 Nacl made in a 50 mL volumetric flask
- Prepare a standard concentration of 50 μ g/mL by adding 5mL of diluted *o*-nitrophenyl solution (1mg/mL) to 95 mL of 0.7 Nacl made in a 100 mL volumetric flask
- Prepare a standard concentration of 80 μ g/mL by adding 4mL of diluted *o*-nitrophenyl solution (1mg/mL) to 46 mL of 0.7 Nacl made in a 50 mL volumetric flask
- Add (for control) 1000 μ L of unfermented soymilk in a clean dry test tube. Add 1000 μ L of *o*- nitrophenol solution (1mg/ml) into a clean dry test tube to act as a blank
- Fermented /unfermented soymilk – 1000 μ L in a clean dry test tube
- Add 1000 μ L of ONPG, incubate for 15 min at 37°C
- Add 500 μ L of cold NaCo₃
- Add 1000 μ L of Nacl for control (Buffer + sample control)
- Add 1000 μ L of Nacl for blank (Only the buffer)

- Take absorbance readings using spectrophotometer at 400nm setting blank as a reference point.
- Draw a linear curve using regression analysis by plotting absorbance of *o*-nitrophenyl verses the concentration

Assay for β -galactosidase activity in soymilk

- Take 10 mL of aliquot (fermented soymilk) in a sterilised bottle
- Add 1000 μ L of 5mM (0.005M) *O*-Nitrophenyl B-D-galactopyranoside to the aliquot and incubate at 37°C for 15 minutes
- Stop the reaction by adding 500 μ L cold 1 M of sodium carbonate (Na₂CO₃)
- Measure the absorbance readings at 400nm using a spectrophotometer.
- Convert readings into enzyme activity, which is the amount of *o*-Nitrophenol released in μ mole per min at 37°C.

Preparation of a blank

- Take 10 mL of aliquot (unfermented soymilk) in a sterilised bottle
- Add 500 μ L of 5mM (0.005M) *O*-Nitrophenyl B-D-glucopyranoside and 500 μ L of *O*-Nitrophenyl B-D-galactopyranoside to the aliquot and incubate at 37°C for 15 minutes
- Stop the reaction by adding 500 μ L cold 1 M of Sodium carbonate (Na₂CO₃)
- Measure the absorbance readings at 400nm using a spectrophotometer.
- Set the obtained reading as reference (Zero/Blank)
- Begin to take sample reading after setting the reference using blank.

- Convert readings into enzyme activity, which is the amount of *p*-Nitrophenol released in μ mole per min at 37°C

C. Quantification of isoflavone compounds found in fermented and non-fermented soymilk samples using HPLC with an internal standard (ISTD)

C.1.0 Preparation of a standard linear curve and equation

Six mixed isoflavone standards each containing daidzein, genistein and glycitein at equal concentration (20, 50, 80 and 100 ng per 10 µL injection volume) and 600 ng of flavone (Internal standard) were analysed using HPLC at the beginning and end of each HPLC run of soymilk analyses. The chromatogram peak area responses for each of these isoflavone standards were used to prepare a standard linear curve and equation to quantify the unknown concentration of isoflavone in each soymilk sample. A blank containing only 600 ng of flavone was also analysed at the beginning and end of each HPLC run.

C.1.1. Calculate the Amount Ratio for each isoflavone standard at each level of calibration (using four-level multi calibration is advised):

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

C.1.2. Calculate the Peak Area Response Ratio for each standard at each level of calibration:

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

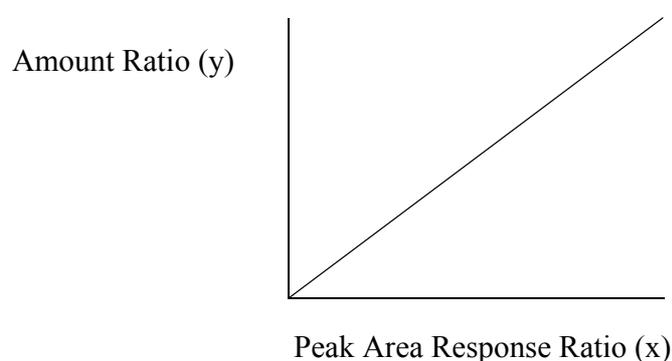
C.1.3. Tabulate the Amount Ratio with its respective Peak Area Response ratio. It is advised to use isoflavone standard concentrations of 20, 50, 80 and 100 ng / 10 uL injection volume (ie. four level multi-calibrations). The internal standard of flavone should be 600 ng/ 10uL injection volume, and added to each mixed standard at this concentration.

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

C.1.4. Plot the Amount ratios and Peak Area Response Ratios on an x, y axis as follows:

Multi-level calibration:

20, 50, 80 & 100 ng /10uL



C.1.5. Determine the line of best fit and linear equation for the curve ($y = mx + c$). Calculate the R^2 or coefficient of correlation (should be between 0.9 and 1.0).

$$\text{Amount Ratio} = \text{RF} \times (\text{Peak Area Response Ratio}) + c$$

Where:

RFx = gradient = Response Factor for values of x

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

The linear equation calculated above will be used to determine the unknown concentrations of isoflavone compounds in the fermented and non-fermented soymilk samples.

C.2.0 Quantifying the unknown isoflavone concentrations found in the soymilk samples.

C.2.1. Calculate the Peak Area Response Ratio for isoflavones detected in the soymilk sample chromatograms

$$\text{Peak Area Response ratio} = \frac{\text{Peak Area Response of detected isoflavone isomer}}{\text{Peak Area of ISTD incorporated into sample}}$$

C.2.2. Calculate the Amount Ratio for Isoflavone detected in the soymilk sample chromatograms using the equation in 1.5 expressed as:

$$\text{Amount Ratio} = \text{RFx (Peak Area Response Ratio for detected isoflavone in sample)} + c$$

C.2.3. Calculate the amount of isoflavone in the 10 uL injection volume (ng) using the following equation:

$$\text{Amount Ratio of detected isoflavone} \times \text{Amount of ISTD* (ng)} = \text{_____ ng/10 uL inj vol}$$

The amount of ISTD will be 600 ng (per 10 uL injection vol).

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

$$\text{_____ ng/10 uL} \times 100 = \text{_____ ng/1 mL resuspension} = 1 \text{ mL aliquot}$$

$$\times 100 = \text{_____ ng/freeze dried soymilk dissolved in 100 mL}$$

C.2.5. Express back to wet basis (ng/100 mL soymilk) by using the volume of soymilk freeze dried to 1.00 g and multiply by the appropriate freeze dry factor:

$$\text{_____ ng/1.00 g freeze dried soymilk} \times (100 / \text{Volume of soymilk freeze dried to 1 g sample})$$

C.2.6. Express as mg isoflavone/100 mL of soymilk by dividing by a factor of 10^6 .

D. Method for calculating the concentration of Malonyl- and Acetyl-Glycoside isoflavone compounds in soymilk analysed with HPLC

D.1.0 Preparation of standard linear curve and equation

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

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

E.g

PAR of daidzin \times (MW of daidzin / MW of malonyldaidzin) = PAR of malonyldaidzin

D.1.2. Calculate the Amount Ratio for each isoflavone β -glycoside standard at each level of calibration:

$$\text{Amount Ratio} = \frac{\text{Concentration of isoflavone } \hat{\alpha} \text{- glycoside standard (ng)}}{\text{Concentration of internal standard (ng)}}$$

D.1.3. Calculate the Peak Area Response Ratio for each malonyl- and acetyl-glycoside compound PAR calculated in section D.1.1

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

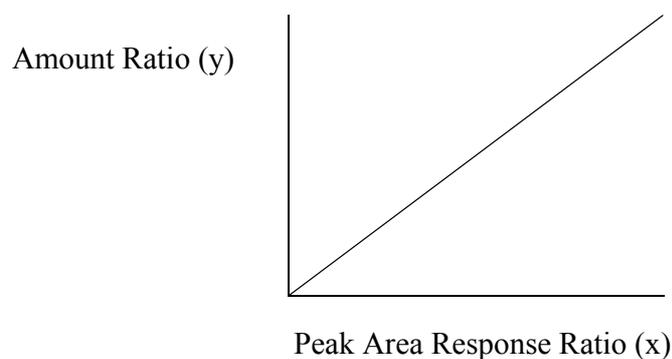
D.1.4 Tabulate the Amount Ratio with its respective Peak Area Response Ratio for each malonyl- and acetylglycoside isomer at each level of calibration.

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

D.1.5. Plot the Amount Ratio and Peak Area Response Ratio for each malonyl- and acetyl-glycoside isoflavone isomer on an x, y axis as follows:

Multi-level calibration:

Blank, 20, 50, 80 & 100 ng /10uL



D.1.5. Determine the line of best fit and linear equation for the curve ($y = mx + c$).

$$\text{Amount Ratio} = \text{RF} \times (\text{Peak Area Response Ratio}) + c$$

Where:

RFx = gradient = Response Factor for values of x

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

The linear equation calculated above will be used to determine the unknown concentrations of isoflavone compounds in the fermented and non-fermented soymilk samples.

D.2.0 Quantification of the unknown isoflavone concentrations in the soymilk samples.

D.2.1. Calculate the Peak Area Response Ratio for each malonyl- and acetyl-glycoside isoflavone detected in the soymilk.

$$\text{Peak Area Response ratio} = \frac{\text{P A R of detected malonyl- or acetyl- glycoside isomer}}{\text{PAR of of internal standard}}$$

D.2.2. Calculate the Amount Ratio for each malonyl- and acetyl-glycoside isomer detected in the soymilk sample chromatograms using the equation in D.1.6 expressed as:

D.2.3. Calculate the amount of isoflavone in the 10 uL injection volume (ng) using the following equation:

$$\text{Amount Ratio of detected isoflavone} \times \text{Amount of ISTD}^* \text{ (ng)} = \text{_____ ng/10 uL}$$

The amount of ISTD = 600 ng (per 10 uL injection vol).

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

$$\text{_____ ng/10 uL} \times 100 = \text{_____ ng/1 mL resuspension} = \text{_____ ng per 5 mL aliquot}$$

$$\text{_____ ng per 5 mL aliquot} \times 20 = \text{_____ ng per 1.00 g freeze dried soymilk in 100 mL}$$

D.2.5. Express back to wet basis (ng/100 mL soymilk) by using the volume of soymilk freeze dried to 1.00 g (~25 mL) and multiply by the appropriate freeze dry factor:

_____ ng/1.00 g freeze dried soymilk x (100 / Volume of soymilk freeze dried to 1 g sample).

D.2.6. Express as mg isoflavone/100 mL of soymilk by dividing by a factor of 10^6 .