

Influence of Polyphenols on the Survival and Adhesion of Probiotic Bacteria

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Influence of Polyphenols on the Survival and Adhesion of Probiotic Bacteria

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“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale”

Marie Curie (1867 -1934)

ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material to which to a substantial extent has been submitted for the award of any degree or diploma of a university or other institution of higher learning.

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ABSTRACT

Functional foods containing probiotic bacteria (PB) and polyphenol (PP) antioxidants are gaining increased market leverage. Many probiotic strains, however, exhibit low viability, because of exposure to high acidity and oxidative stress during industrial processing, storage and gastric transit. This subsequently affects their adhesion to the gastrointestinal (GI) tract. The survival of PB in foods and their attachment to the GI tract are vital for conferring the desired health benefits of PB. The health-benefits of PPs, as well as their ability to reduce oxidative stress in foods, are well recognised. PPs are also sensitive to environmental factors, including heat, oxygen and light. It is of great interest to establish a combination of PPs and PB for delivering their maximised health-promoting properties.

The main objective of this study was to investigate the efficacy of apple skin PPs on the adhesion and survival of PB in a model milk drink system. Apple skin PP extracts, prepared using either ethanolic or aqueous extraction methods, were used as sources of PPs. These two apple skin PP extracts were analysed in terms of their total extractable polyphenol content (TEPC), total antioxidant activity (TAA), pectin fibre and vitamin C contents. To elucidate the effect of individual PP compounds on the viability of PB, both the crude apple skin PP extracts and commercially available purified PP compounds were added to milk drinks containing PB *Lactobacillus acidophilus*, which were then subjected to 50-day storage at 4° C. The influence of the two apple skin extracts and purified PPs on the adhesion of *L. acidophilus* was evaluated using a crystal violet attachment assay that utilised hydrophilic tissue culture treated microtitre plates as the attachment surface. Co-extrusion technology was also used to microencapsulate PB alone and co-encapsulate PB and either of the two apple skin PP extracts, aiming to preserve these bioactive components in the final milk drinks. Finally, the effect of an acidic condition (pH 2 for 120 minutes, simulating the exposure of foods to the stomach environment) on the integrity of the encapsulated beads containing PB alone or the combination of PB and PPs, was investigated. Results indicated that the aqueous apple skin extract had significantly higher TEPC (21.44 mg catechin eq./g extract) and TAA (25.69 mg Trolox eq./g extract) compared with ethanolic extract (with TEPC, 20.66 mg catechin eq./g extract; TAA, 23.98 mg Trolox eq./g extract). The pectin fibre and vitamin C contents of the two extracts were statistically similar. The two apple skin PP extracts (at a concentration of 1% or 2%) and the six purified PP compounds were remarkably effective in maintaining the viability of PB in the milk drinks above the required

minimum PB level (10^6 CFU/mL) after 50-day storage. In contrast, the viability of *L. acidophilus* gradually declined in the control milk (in the absence of added PPs) to 5.91 Log CFU/mL on Day 50 of the refrigerated storage. The apple skin PP extracts and purified PPs were found to be effective in improving the adhesion of PB to the surface of the hydrophilic microtitre plate. The aqueous apple skin extract promoted significant adhesion of PB under both aerobic and anaerobic conditions and at both incubation temperatures (37 and 4 °C). Microencapsulation of PB and especially co-encapsulation of PB with an apple skin PP extracts exerted remarkable ability to prolong the survival of PB in the milk. Cell loss of 0.13-Log CFU/mL and 0.16-Log CFU/mL was detected when PB was co-encapsulated with the aqueous and ethanolic apple skin extracts, respectively. In comparison, a decrease of 1.1-Log CFU/mL in cell count was detected for the unencapsulated PB.

This thesis contains six chapters. Chapter 1 is the introduction, describing the significance of this research topic, the objectives of the study and the research strategy used. Chapter 2 provides the review of the literature mainly related to PB and PPs. Chapter 3 covers the preparation and physicochemical analyses of the ethanolic and aqueous apple skin extracts. Chapter 4 evaluates the impacts of the two apple skin PP extracts and purified polyphenol compounds on the viability and adhesion of probiotic *L. acidophilus*. Chapter 5 presents a new delivery technology i.e. co-extrusion encapsulation and its efficient protection of PB and PPs. The effect of an acidic condition (pH 2 for 120 minutes) on the integrity of the encapsulated beads with PB alone or the combination of PB and PPs was investigated in comparison with unencapsulated *L. acidophilus* cells. Chapter 6 summarises the main findings of this study, indicating the positive and significant effects of apple skin PP extracts on maintaining the viability of PB and improving their adhesion ability. Finally, future research directions have been suggested.

The knowledge obtained from thesis not only contributes to scientific advances, such as the positive influence of apple skin PP extracts on the viability and adhesion of PB, but also demonstrates the commercial potential of a concept for a stable milk drink containing sufficient and desired PB and apple PPs.

INTELLECTUAL PROPERTY RIGHTS

The work contained in this thesis could potentially be commercialisable and the author and her two supervisors claim intellectual property rights over this research.

LIST OF PRESENTATIONS

This work has been presented in part in following poster presentations at the scientific conferences:

1. Shinde, T., Brooks, J., & Sun-Waterhouse D. (2011). Influence of the polyphenol extracts from apple skin on the viability of probiotic bacteria in model milk drink. *Functional Foods Symposium*, Auckland, New Zealand. **Poster presentation** (Appendix I) (Awarded 1st prize)
2. Shinde, T., Brooks, J., & Sun-Waterhouse D. (2012). Influence of the polyphenol extracts from apple skin on the viability and adhesion of probiotic bacteria in model milk drink. *NZIFST conference*, Hamilton, New Zealand & *AUT Postgraduate Symposium*, Auckland, New Zealand. **Poster presentation** (Appendix II) (Awarded 3rd prize at the NZIFST conference)

DEDICATION

I wish to dedicate this thesis to the most loving people in my life – my husband Sandesh and my parents Meena and Govind for their endless love, support and encouragement. Thank you all for giving me the strength to reach for the stars and chase my dreams. My parents not only raised and nurtured me in the best possible ways but have always encouraged and supported my interests throughout. Completing this degree meant a huge amount to me since my mother never had the chances that I have been given. Thanks Sandesh for being the source of motivation and strength during moments of despair and difficulties. Your love and care have been shown in incredible ways. Thank you for believing in me more than I do.

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Scott Adams (Dilbert) said, “You don't have to be a "person of influence" to be influential. In fact, the most influential people in my life are probably not even aware of the things they've taught me”. I was fortunate to have enjoyed the support of a number of people throughout the course of my MSc degree study without whom this thesis would have been impossible. I am grateful to all of them who have contributed towards this thesis directly or indirectly.

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ABBREVIATIONS

Abbreviation	Meaning
AA	Antioxidant activity
ANOVA	Analysis of variance
ASE	Accelerated solvent extraction
CFU	Colony forming units
CO₂	Carbon dioxide
ESEM	Electron scanning microscopy
FAO	Food and Agriculture Organisation of the United Nations
FDA	Food and Drug Administration
FRAP	Ferric Reducing Antioxidant Power
g	Gram
GaLA	Galacturonic acid
GI	Gastrointestinal
GRAS	Generally regarded as safe
H₂O₂	Hydrogen peroxide
H₂SO₄	Sulfuric acid
h	Hour
HepG2	Liver hepatocellular carcinoma
HPLC	High Performance Liquid Chromatography
HSD	Honestly Significant Difference
IBS	Irritable Bowel Syndrome
Ig	Immunoglobulin
IL	Interleukin
L	Litre
LAB	Lactic acid bacteria
LPH	Lactase phlorizin hydrolase
mAU	Milliabsorbance units

ME	Microencapsulation efficiency
min	Minute
mL	Millilitre
MPa	Megapascal
MRS	deMan Rogosa & Sharpe
NaOH	Sodium hydroxide
NK	Natural killer
O D	Optical density
PB	Probiotic bacteria
PP	Polyphenol
RO	Reverse osmosis
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
SEM	Scanning electron microscope
SGLT	Sodium-dependent glucose transporter
TAA	Total antioxidant activity
TEPC	Total extractable polyphenol content
UA	Uronic acid
U.K.	United Kingdom
WHO	World Health Organisation
w/v	Weight by volume
w/w	Weight by weight
μ	Micro

Chapter 1

Introduction

1.1 Significance of this Topic

1.1.1 Problem background and the purpose of the study

Consumers' perspective on food is changing which leads to the demand for foods possessing health benefits beyond basic nutrition. Nowadays, food is no longer a source of essential nutrients to satisfy human hunger, but also, an important vehicle carrying health-promoting bioactives to reduce the risk of diseases. This trend directs research towards the development of foods for optimal wellness (Menrad, 2003, Yan et al., 2009). Therefore, functional foods carrying probiotic bacteria (PB) and plant phytochemicals like polyphenol (PP) antioxidants may become an important category of functional foods (Hasler, 1998, Menrad, 2003, Shanahan, 2003). The soaring healthcare costs, gradual increased life expectancy, desire of a healthier life, rigorous food legislation and advances of technologies all drive the development of functional foods (Korhonen, 2002, Roberfroid, 2000).

The emergence of antibiotic resistant pathogens and the requirement for natural means to suppress pathogenic microorganisms have brought the concept of “probiotics” in the spotlight (Goldin and Gorbach, 1984). Probiotics are defined as “live micro-organisms which, when, administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2001). The balancing of intestinal microflora with probiotic therapy is claimed to be a good approach for prevention or treatment of several ailments including infectious and inflammatory conditions as well as gastro intestinal dysfunctions (Kailasapathy and Chin, 2000, Shanahan, 2003). Moreover, the apparent safety of probiotic therapy relative to drug therapy appeals to the consumers' acceptance to probiotic food (Shanahan, 2003). The viability of PB in the products throughout the shelf-life before consumption and the journey along the digestive tract, as well as their efficient attachment to colonic cells, governs the ultimate therapeutic properties of probiotic foods (Kailasapathy and Chin, 2000). The minimum effective dose of PB per day was proposed to be 10^8 - 10^9 cells per gram of food item. Similarly, foods merchandised with any health-promoting claims should at least satisfy the minimum

requirement of 10^6 colony forming units (CFU)/mL probiotic cells until the expiry date (Kailasapathy and Chin, 2000, Kurmann and Rasic, 1991). Many PB strains however, have been found to be unable to withstand the high acidity and oxidative stress to which bacteria would possibly be exposed during industrial processing, handling, storage and gastric transit (Iwana et al., 1993, Shah and Jelen, 1990, Shah et al., 1995, Varnam and Sutherland, 1994). Low viability of PB in food would subsequently cause inefficient adhesion of PB to colonic cells. Viability and attachment properties of PB are two essential factors for any examination of PB's health benefits on the host (Mattila-Sandholm et al., 2002, Shanahan, 2003, Stanton et al., 2003). Maintaining the viability of PB in food products is possibly the first basic requirement for probiotic manufacturers.

Polyphenols (PPs), on the other hand, are well recognized antioxidants that can scavenge free radicals and reduce the oxidative damage in foods or biological systems like human bodies (Kaur and Kapoor, 2001). PPs are secondary metabolites synthesised by vascular plants that play a variety of roles ranging from structural to protection (Stalikas, 2007). A number of studies have demonstrated the antioxidant capacity of PPs from a wide variety of plant materials including fruits (Velioglu et al., 1998, Vinson et al., 2001, Wolfe et al., 2003), vegetables (Ou et al., 2002, Vinson et al., 1998) and grain products (Velioglu et al., 1998) as well as from plant-derived foods including dried fruits (Vinson et al., 2005), chocolate (Vinson et al., 1999) and beverages (Pellegrini et al., 2003, Seeram et al., 2008). The antioxidant capacity of PPs has been associated with the lower risk of many diseases including cancer, cardiovascular, inflammatory and neurodegenerative diseases (Boyer and Liu, 2004, Hervert-Hernandez and Goni, 2011, Luximon-Ramma et al., 2002).

Owing to the inability of PB to survive under high oxidative stress and high acidity in the food during refrigerated storage, the approach of enhancing their functionality using antioxidant PPs offers an attractive alternative to the conventional acidic foods like yoghurt (Koren et al., 2009). Moreover, utilization of PPs possessing antioxidant power to selectively trigger the growth and activity of PB has been suggested (Koren et al., 2009). Pronounced efficacy of polyphenols to enhance the proliferation and adhesion of probiotic bacteria has been demonstrated (Parkar et al., 2008). This indicates the possibility to formulate a probiotic food with improved adhesion and resistance to environmental stress, and increased PB colony numbers and PP content in the final products by exploiting the synergism between PB and PPs.

This study utilized the PPs from apple skin to evaluate its efficacy in influencing the viability and adhesion of PB. The results of this study could identify apple skin as a potential prebiotic ingredient rich in antioxidant PPs to be used for dairy products containing PB. Furthermore, the results could be applied to design a novel functional dairy drink containing the health promoting goodness of both probiotics and polyphenols.

1.1.2 Commercial value

Dairy products fortified with PB have emerged as one of the most successful segments of the functional food market (Prado et al., 2008). The dairy industry has designed a variety of products to deliver PB in convenient forms including fermented and non-fermented milk, yoghurt, drinking yoghurt, ice cream and desserts (Shah, 2000). Value-added production offers potential alternative to boost the sales of functional foods hence, the development of functional foods has moved towards incorporating food additives that may confer positive effect on the gut microbiota (Ziemer and Gibson, 1998). The functional foods market is widespread and is rapidly mushrooming, particularly in North America, Europe and Asia. The functional dairy food and ingredient market worldwide represents a prominent sector of this general functional food market and is also showing immense growth (Playne et al., 2003). United states, Europe and Japan accounted for about 33.6%, 28.2% and 20.9% of sales in 2003 respectively making them the largest markets for functional foods and supplements (Granato et al., 2010a). Functional dairy products have been reported to increase the market volume in Germany from approximately \$5 million in 1995 to \$419 million in 2000, of which pre/probiotic and other functional yoghurt accounted for about \$301 million, while \$118 million for functional drinks (Menrad, 2003). In North America about 19% of adults purchased pre/probiotic yoghurt compared with only 11% in 2006 as reported by Granato et al. (2010a). The Europe leads the probiotic market of fermented milk products (including yoghurt) with annual per-capita consumption of 35-45 L/person/year) compared with only 4-5 L/person/year per-capita consumption in North America (Saxelin, 2008). According to the report by Shelke (2009) the sales of probiotic yoghurt doubled to \$13.7 billion in 2008 from just \$6.8 billion in 2003 and is expected to double gain by 2013. Japan is recorded to lead the battle with per capita consumption of about 8.7 lb, followed by Europe with 4.7 lb and the US with 1.8 lb. The demand and knowledge of probiotic food assisted health benefits among consumers is driving the functional foods market rapidly.

Inclusion of fruits in the diet on a regular basis has been documented to reduce the incidences of chronic and infectious diseases including cancer and cardiovascular diseases (Block et al., 1992, Riboli and Norat, 2003, Rimm et al., 1996, Vatter et al., 2005). The antioxidants and dietary fibre of the fruits have been shown to account for these health promoting and protective properties (Arts and Hollman, 2005, Bravo et al., 1994, Middleton et al., 2000, Scott et al., 2008). There exists a huge demand for functional foods containing the goodness of fruits because of their perceived “naturalness” and their diverse nutrient composition (Sun-Waterhouse, 2011).

Apples are one of the most common fruits consumed on a regular basis by many cultures (Boyer and Liu, 2004). Apples are a significant source of PP antioxidants and 22% of dietary phenolics have been estimated to be consumed from apples (Vinson et al., 2001). The antioxidant polyphenols from apple have been linked to reduced incidences of cancer (Feskanich et al., 2000, Marchand et al., 2000), cardiovascular diseases (Arts et al., 2001, Knekt et al., 2000) and asthma (Shaheen et al., 2001, Woods et al., 2003), as well as lowered risk of diabetes and weight loss (Conceicao de Oliveira et al., 2003, Knekt et al., 2002).

So formulation of a probiotic dairy drink fortified with apple PPs may offer an opportunity to provide value added functional benefits in addition to providing an opportunity for the influencing the viability and attachment of PB.

1.2 Research Strategy

This thesis aims to explore the effect of PP extracts from apple skin extracted using either ethanolic or aqueous extraction methods on the adhesion and viability of PB in model fruit extract enhanced- milk drinks. The rationale behind the choice of apple skin PPs and milk, experimental approach and experimental conditions is outlined below.

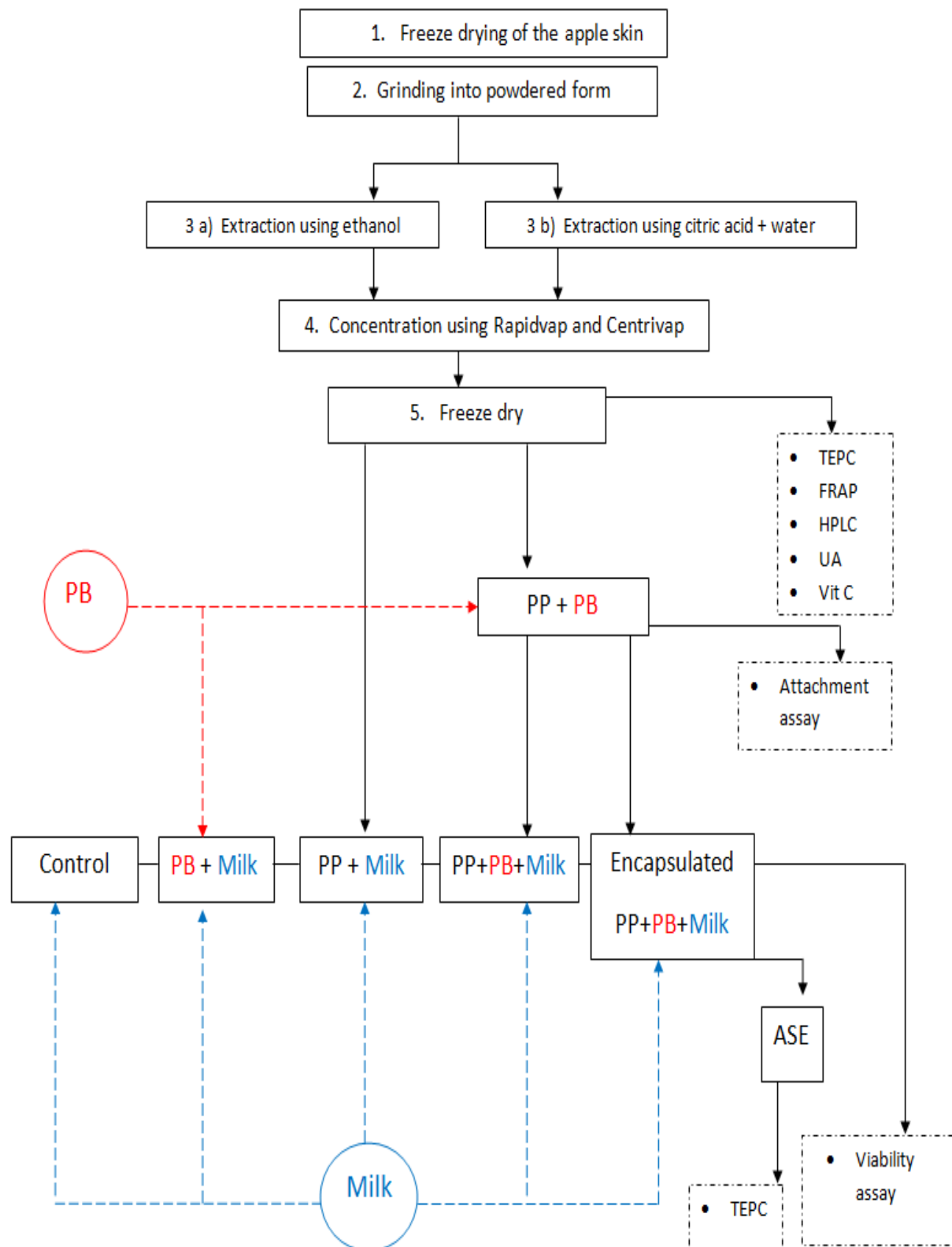
The desire to study the interaction between PPs and probiotics stems from the fact that both of these components have been well established to confer health- promoting effects individually. Moreover, PPs have been shown to influence the bioactivity (proliferation and adhesion) of beneficial bacteria (Koren et al., 2009, Parkar et al., 2008, Sun-Waterhouse et al., 2011e) and to inhibit pathogens (China et al., 2012, Su et al., 2008) at a given concentration. This allows a possibility to produce bacterial cells that can tolerate environmental stresses, encountered during the production of functional foods and during gastric transit. The synergism between PPs and PB has presented an opportunity to design functional food that will deliver the goodness of both these constituents through a convenient food format. These interactions may be invigorating to human health and hence, elucidating these interactions may aid in optimising the beneficial synergism, ultimately leading to successful formulation of functional foods or improved eating practices.

Apples are one of the most commonly consumed fruits and a rich source of PPs thus, they were the logical choice for this study. Apple skin has a higher concentration of PPs than its flesh, and hence its utilization in this study provides an alternative for its usage as a potent antioxidant ingredient, instead of wasting it. The PPs from apple skin was extracted using either ethanol or acidic water. Ethanol and water as extraction solvents were chosen in this study with a view of using non-toxic, cheap and readily available means of recovering PPs from apple skin waste. Ethanolic and aqueous preparative methods of PPs represent procedures similar to those used in the PP manufacturing industry, and hence, the results achieved in this study might provide a more specific indication of the application of apple skin as a food ingredient. The prepared extracts were then subjected to various analyses including Folin-Ciocalteu assay and Ferric Reducing Antioxidant Power Assay (FRAP) to determine the total extractable polyphenol content and antioxidant capacity of the skin extract respectively. The vitamin C and dietary fibre contents of the apple skin PP extracts were also examined.

The milk was used as a food system to evaluate the efficacy of the skin PP extracts in maintaining the viability of probiotic *Lactobacillus acidophilus*. Milk is a significant provider of vital nutrients, to humans. Moreover, the simplicity of its matrix, relative to other complex dairy products like yoghurt, made it a suitable medium for use in this study to understand the interaction between PB and PPs without interference from fermentation induced components. PP enhanced probiotic milk was stored at 4 °C for 50 days and the viability of PB was assessed every 5 days. The storage temperature (4 °C) was chosen keeping in mind the normal refrigeration temperature used in the supermarkets to store dairy products like drinking yoghurt, milk etc.

The adhesion assay was performed with a crystal violet absorption assay in hydrophilic tissue culture treated microtitre plates that provided an attachment surface representing the gut lining. The incubation of PPs and PB for the adhesion assay was performed under conditions similar to those encountered in the human gastrointestinal tract. The choice of MRS medium rather than milk for this assay was made in order to avoid the attachment and interference from milk proteins (covalent binding). MRS contains only 1% of peptone and the manufacturers claim that peptone contains only a negligible amount of proteoses and other complex constituents. Hence, there will be negligible to no binding effect of PPs to proteins in this study. This study also employed commercial polyphenol compounds to understand and compare the effect of individual PPs compared with the crude PP extract, composed of varied polyphenol species, on the adhesion and viability of PB.

The microencapsulation technique was employed in the study to examine its influence in preserving both PPs and PB in the milk system. The extrusion microencapsulation technique used in this study was chosen keeping in mind the simplicity of the method, cost-effectiveness and gentle operations which minimizes cell injury. PB and apple skin PP extracts were microencapsulated in alginate polymer beads. These beads were then added to milk that underwent a similar storage trial at 4 °C for 50 days. Microscopic examinations were performed to determine the ability of alginate polymer to trap the PB in the matrix. Alginate was employed as an encapsulant for its ease in forming gel matrices around bacterial cells, its non-poisonous nature, cost-effectiveness and ease of handling the gel beads. No oil was used during the microencapsulation process in this study, which may be a plus point in regards to sensory perception. An overview of the experimental program undertaken is shown in Figure 1.1.



PP = Polyphenols, PB = Probiotic bacteria, TEPC = Total extractable polyphenol content, FRAP = Ferric Reducing Antioxidant Power Assay, HPLC = High Performance Liquid Chromatography, UA = Uronic acid, ASE = Accelerated Solvent Extraction

Figure 1.1. Experimental overview

1.3 Objectives of the Thesis

The overall aim of this study was to investigate the influence of apple skin polyphenols extracts on the activity of probiotic bacteria.

The detailed objectives were:

- To determine the influence of apple skin polyphenol extracts prepared by using two different extraction methods on the viability of probiotic bacteria in model milk drink.
- To determine the effect of the two apple skin polyphenol extracts on the adhesion of probiotic bacteria.
- To evaluate the efficacy of co-encapsulation of probiotic bacteria with apple skin polyphenols on the probiotic viability in mode milk drinks.

1.4 Thesis Overview

Chapter 2 reviews the literature associated with probiotic bacteria and apple polyphenols. The importance of viability and adhesion of probiotic bacteria in order to receive the targeted health benefits is also emphasised. In addition, this chapter also highlights the technological challenges encountered during the production of probiotic food as well as the means to encounter these challenges. The antioxidant polyphenols in apples and their impact on health is described. Finally, the studies associated with probiotic bacteria and polyphenols are discussed.

Chapter 3 provides the comparison between the ethanolic and aqueous extraction methods used to extract polyphenols from apple skin. The two extraction solvents are compared for their ability to generate best quality extracts in terms of their total extractable polyphenol content, antioxidant activity, and dietary fibre pectin and vitamin C contents. The polyphenolic content of the model milk drinks fortified with either ethanolic or aqueous apple skin extracts is also examined.

Chapter 4 covers the evaluation of the effect of the apple skin polyphenol extracts prepared by ethanolic or aqueous extraction methods on the activity of probiotic bacteria. The influence of the two apple skin polyphenol extracts on the viability of probiotic bacteria *Lactobacillus acidophilus* in refrigerated milk drinks is examined. The effect of these extracts on the adhesion of probiotic bacteria is also studied.

Chapter 5 presents the investigation of the efficacy of co-extrusion microencapsulation to provide protection to these beneficial microorganisms during cold storage in milk drinks and in acidic medium. Co-encapsulation of probiotics with apple skin polyphenol extracts to enhance the viability of probiotic bacteria in milk drinks and their survival in acidified water is also analysed.

Chapter 6 summarises the results from Chapter 3 to 5 and the different aspects of the thesis were considered as a contribution to the existing literature associated with the interaction between polyphenols and probiotics. Limitations in the project and the potential future research directions are also discussed.

Chapter 2

Review of the literature

2.1 Probiotic bacteria

2.1.1 Changing perception of probiotic bacteria

The probiotic concept stems from the work of Metchinkoff at the beginning of the 1900s. According to him, the complex microflora in the colon was negatively affecting the host through “autointoxication” and he claimed that Bulgarian peasants who consumed large amounts of fermented milk, experienced longevity. This was associated with health enhancing properties of live organisms. Probiotic bacteria (PB) have been defined by many authors in numerous ways, depending on their understanding of mechanisms of action and their effect on well-being of the host. But perhaps the most widely used and accepted definition is addressed by Fuller (1992) according to whom, probiotics are living micro-organisms, which upon ingestion beneficially affects the host by improving the balance of the intestinal microflora. The term “probiotic” is originally derived from Greek, meaning ‘for life’ and is traditionally used to describe the use of live micro-organisms as food supplements that benefit the host through improvement of intestinal microbial balance (Fuller 1989). Havenaar and Huis (1992) redefined probiotics as “a preparation of, or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host”. Yet another definition from Tannock et al. (2000) describes probiotic bacteria as “live microbes that transit the gastro-intestinal (GI) tract and in doing so benefit the health of the consumer”, which contradicts the earlier definitions that highlight only the interactions between the probiotic organisms and the indigenous intestinal microflora. FAO/WHO in 2001 redefined the term probiotics as “live micro-organisms which, when, administered in adequate amounts, confer a health benefit on the host”. All these definitions agree on one point that probiotics should impart health benefits and should be living in nature and are able to colonise the intestine.

Although there is increasing evidence that probiotics can benefit the human host by acting as a first line of defence against disease-causing pathogens by improving the intestinal microflora, for a particular bacterial strain to be categorised as probiotic in nature, it must fulfill certain criteria (Saarela et al., 2000). These include the physiological and manufacturing demands through which the bacterial strain must survive, be safe and also have all the capabilities to exert the beneficial effects on the host as per their basic definition. A probiotic microorganism is anticipated to promote the health of the host in general, but to critically define a particular strain as probiotic, the following three categories of key criteria have been identified, that are desirable in any probiotic strain (Kailasapathy and Chin, 2000, Saarela et al., 2000). Figure 2.1 illustrates the three categories of key criteria for the selection of probiotic microorganisms: Safety, functional and technological characteristics.

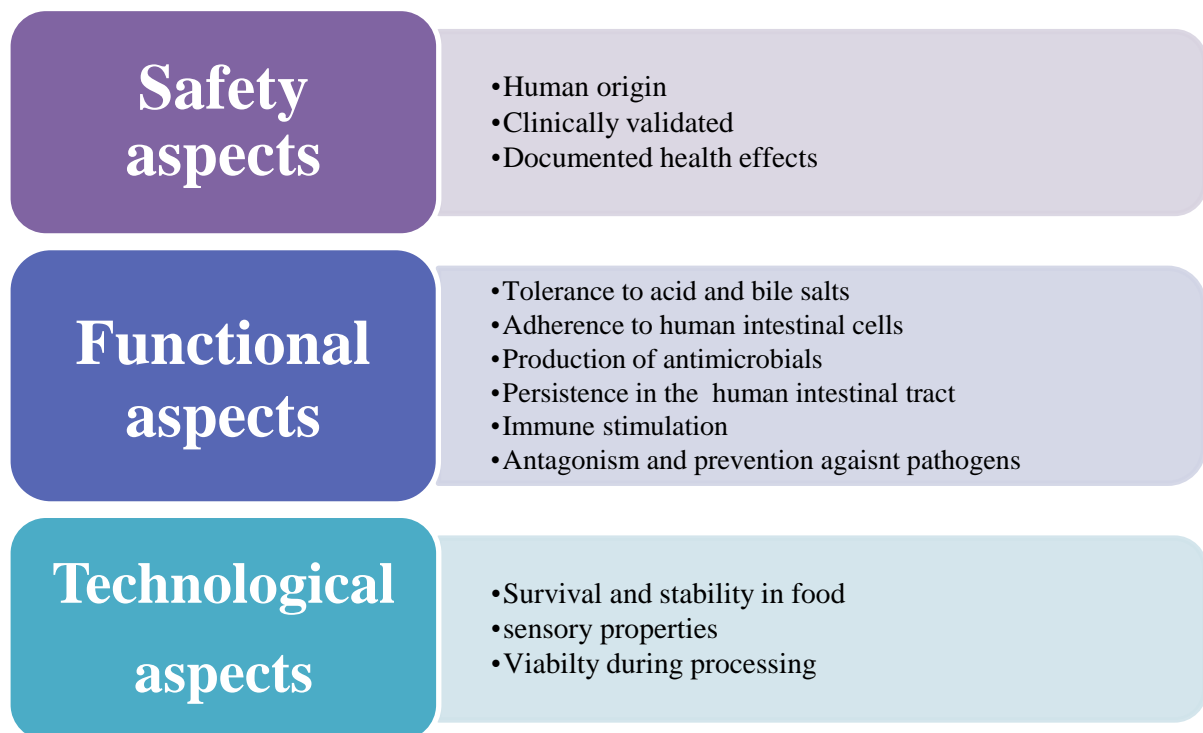


Figure 2.1. The key criteria for selection of probiotic microorganisms (After Saarela et al., 2000)

2.1.2 Probiotics in human gastro-intestinal tract

Human gastro-intestinal (GI) tract microflora represents an ecosystem with high complexity and understanding of this system and its interaction is still limited. The GI tract of an adult human is estimated to harbour about 10^{14} viable bacteria (Berg, 1996, Holzapfel et al., 1998). Figure 2.2 illustrates the colonisation of human GI tract with indigenous microflora. The oral cavity, which marks the beginning of GI tract is made up of mouth, nose and throat and is known to harbour a complex microbiota of about 200 species. A large number of bacteria have also been reported to colonise the anterior and posterior tongue, sub and supragingival plaque, buccal mucosa and vestibular mucosa (Berg, 1996). The acidic environment inside the stomach contains only a low number of microorganisms of about 10^3 - 10^4 CFU/mL of gastric contents. The small intestine does not provide a healthy environment for the growth of bacteria, owing to a relatively swift flow, low pH and the presence of bile. The same holds true for the duodenum, which secretes intestinal fluids, thus creating a hostile environment for the microbial population. The main microbial species found in the upper small intestine include acid-tolerant lactobacilli and streptococci that survive the passage through the stomach (Berg, 1996). The numbers as well as the variety of probiotic species increase progressively along the jejunum and ileum. The large intestine is the primary site of colonisation for probiotic bacteria, owing to favourable factors such as slow transit time, favourable pH environment and availability of nutrients. The small intestine contains species in the range of 10^4 - 10^6 CFU/mL while this population goes up to 10^{10} - 10^{12} CFU/mL in the large intestine (Berg, 1996, Holzapfel et al., 1998, Nag, 2011).

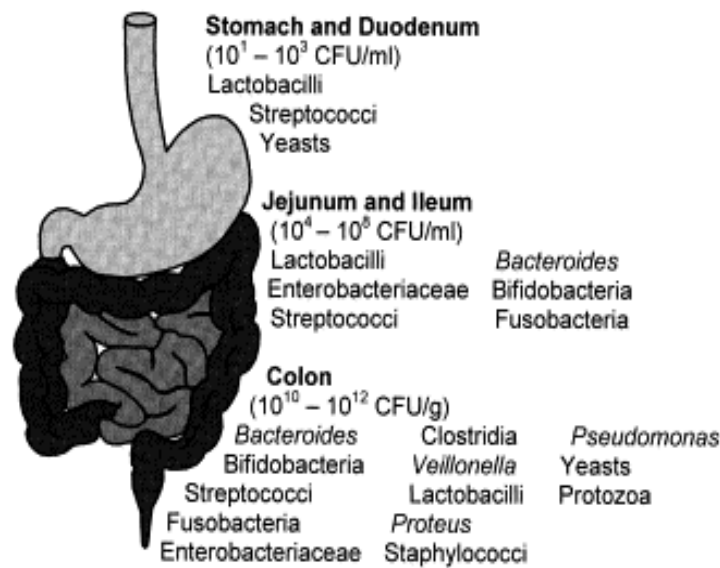


Figure 2.2. Microbial colonisation of human gastrointestinal tract (Holzapfel et al., 1998)

The human GI tract remains sterile until the birth of a newborn and then the microbial colonisation process begins during the delivery process. The inoculum is mainly obtained from the mother's vaginal or faecal flora in case of conventional birth or from the environment during caesarean delivery (Holzapfel et al., 1998). This microbial population gradually evolves in a fashion that can best cope with the physiological and microbiological pressure encountered within the ecosystem. This barrier effect gives the indigenous microbiota the ability to fight against the invading pathogens and to compete for nutrients. The population of *Escherichia coli* and *Streptococcus* that predominates in the very beginning decreases with breast feeding while the *Bifidobacterium* population sharply rises (Adlerberth et al., 2000, Berg, 1996, Holzapfel et al., 1998).

The healthy intestinal epithelium harbouring a good microflora creates a barrier against the uptake of pathogens, antigens and other detrimental compounds from the gut lumen. Intestinal probiotics have been demonstrated to challenge the invading pathogens and prevent infection through competitive exclusion and hence, the composition of the intestinal microflora significantly influences gastrointestinal health (Berg, 1996, Holzapfel et al., 1998, Nag, 2011). The intestinal microflora is also involved in various beneficial activities that include the degradation of certain food components, production of vitamin B and K, synthesis of digestive enzymes and the stimulation of the immune system (Berg, 1996).

2.1.3 Commercially important probiotic species

Presently a large number of species has been recognised as possessing probiotic attributes. Major PB, mainly *Lactobacilli* and *Bifidobacterium*, are of high importance to researchers and the majority of the research has been concentrated on them. This may be because of the perception that they are the desirable members of the intestinal microflora and have “GRAS” (generally regarded as safe) status (Dunne, 2001). Consequently, they have been widely employed in the food industry as probiotic microorganisms. Probiotics are commercially marketed either in a lyophilised form or as fermented food products. *L. acidophilus*, *L. casei* strain Shirota, *L. rhamnosus* and *L. reuteri* are among the most popular choices and have a long application history, followed by some *Bifidobacterium* spp. Also, a few non-lactics are utilised mainly in pharmaceutical preparations (Holzapfel et al., 1998). Other than these bacteria, *Bacillus cereus* var. *toyoi*, *Escherichia coli* strain Nissle, *Propionibacterium freudenreichii*, and some types of yeasts, e.g. *Saccharomyces cerevisiae* and *Saccharomyces boulardii* have also been identified as having probiotic effects (Holzapfel et al., 1998). Table 2.1 shows a list of popular probiotic strains used for various applications. The characteristics and morphology of the two most widely used probiotic species, *Lactobacillus* and *Bifidobacterium* are discussed below.

Table 2.1. Commonly employed probiotic strains (After Holzapfel et al., 1998)

<i>Lactobacillus</i> species	<i>Bifidobacterium</i> species	Other LAB	Non-lactics
<i>L. acidophilus</i> <i>L. casei</i> <i>L. crispatus</i> <i>L. gasseri</i> <i>L. johnsonii</i> (<i>L. paracasei</i>) <i>L. plantarum</i> <i>L. reuteri</i> <i>L. rhamnosus</i>	<i>B. adolescentis</i> <i>B. animalis</i> <i>B. bifidum</i> <i>B. breve</i> <i>B. infantis</i> <i>B. lactis</i> ^b <i>B. longum</i>	<i>Enterococcus faecalis</i> ^a <i>Enterococcus faecium</i> <i>Lactococcus lactis</i> ^c <i>Leuconostoc</i> <i>mesenteroides</i> ^c <i>Pediococcus acidilactici</i> ^c <i>Sporolactobacillus</i> <i>inulinus</i> ^a <i>Streptococcus</i> <i>thermophilus</i>	<i>Bacillus cereus</i> ('toyoi') ^{a,d} <i>Escherichia coli</i> ('Nissle 1917') ^d <i>Propionibacterium freudenreichii</i> ^{a,d} <i>Saccharomyces cerevisiae</i> ('boulardii') ^d

^a Mainly used for animals.

^b Probably synonymous with *B. animalis*.

^c Little known about probiotic properties.

^d Mainly as pharmaceutical preparations.

The genus *Lactobacillus* is characterized as Gram-positive rods, non-sporulating, catalase-negative and oxidase-negative. These bacteria are devoid of cytochromes and non-aerobic in nature but, are aero-tolerant, fastidious, acid-tolerant and strictly fermentative. Lactic acid is the major end-product of sugar fermentation by lactic acid bacteria (LAB) (Anal and Singh, 2007, Axelsson, 2004, Gomes and Xavier, 1999). LAB have been well documented to impart health benefits to humans through immune stimulation, inhibition of pathogens and improved digestion. They have also have a long safe food application history which makes them a popular choice (Stanton et al., 2003). *Lactobacillus* accounted for 61.9% of total sales in 2007 according to the reports by Food Processing (2009). The most common among all species found in the human gut is *Lactobacillus acidophilus* complex, *Lactobacillus salivarius* and *Lactobacillus casei* complex. *Lactobacillus acidophilus* is the most commonly employed LAB for dietary use, owing to its acid and bile tolerant properties, antimicrobial activity and its ability to survive during GI transit (Gomes and Xavier, 1999). *L. acidophilus* can grow at temperatures as high as 45 °C, but the optimum growth occurs between 35-40 °C. The bacteria have the capacity to grow at pH 6.4-4.5 but growth arrest occurs between pH 4.0-3.6 while, the optimum pH of the organism is noted between 5.5-6.0 (Curry and Crow, 2003, Shah, 2007).

Lactobacillus salivarius is known for its acid and bile tolerant ability, good adherence to gastric epithelial cells and smooth transit through intestinal tract. A few other known LAB that are used as probiotics are *Lactobacillus amylovorus*, *Lactobacillus casei*, *Lactobacillus crispatus*, *Lactobacillus delbrueckii*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus* etc. (Mayra-Makinen et al., 1993).

Bifidobacteria is another probiotic species that has been widely used. Bifidobacteria are classified as Gram-positive rods, non-sporulating, catalase-negative and are strictly anaerobic. The optimum temperature for the genus is between 37-41 °C, while the minimum and maximum growth temperatures are 25-28 °C and 43-45 °C respectively. These bacteria have low tolerance to acid and are also unable to grow in media with high oxidative potential. The optimum growth of *Bifidobacterium* occurs between pH 6.0-7.0, while, the growth ceases at pH 4.5-5.0 and below or at pH 8.0-8.5 (Shah, 2007).

Bifidobacteria actively ferment carbohydrates, producing mainly acetic acid and lactic acid, but not carbon dioxide, butyric acid and propionic acid (Anal and Singh, 2007, Stanton et al., 2003). Bifidobacteria are the predominant variety in the infant colon, with *Bifidobacterium longum*, *B. infantis* and *B. breve* dominating, and they are the third most populous in the adult colon with the maximum presence of *B. longum* and *B. adolescentis*, and hence are frequently used as probiotic cultures (Nag, 2011). The most recognised species of bifidobacteria that are commonly employed as probiotic organisms also include *B. animalis*, *B. bifidum* and *B. lactis* (Anal and Singh, 2007, Holzapfel et al., 1998).

2.1.4 Key aspects for evaluating probiotic biological properties

A particular strain is regarded as probiotic if it can confer a certain health benefit on the host. The major and most vital functional benefits obtained in general from a single or a group of probiotic strains include immunomodulation, modulation of intestinal flora and prevention of diarrhoea (Saarela et al., 2000). Probiotics have also been shown to play a therapeutic role by modulating immunity, lowering cholesterol, improving lactose tolerance and preventing some cancers (Kailasapathy and Chin, 2000). Probiotics have also been associated with enhancing digestive and respiratory functions as well as prevention and restriction of infectious diseases in children and immuno-compromised patients (FAO/WHO, 2001). However, the viability of probiotics within the food item and GI tract, their transit and adhesion in the intestine and the fate of probiotic-derived bioactive components are necessary to determine the effects of probiotic consumption. The survival of probiotic strains vary in different parts of GI tract : Some strains are rapidly inhibited while others survive the transit through the gut in high numbers (Marteau et al., 2001, Saarela et al., 2000). The low viability of PB in the gut concurrently affects their adhesion to intestinal cells which lowers the functionality of the probiotic consumption. The adhesion and viability of PB are the two attributes which have gained more weightage when evaluating the optimal functionality of consuming PB (Saarela et al., 2000).

2.1.4.1 Adhesion of probiotic bacteria

Adhesion of probiotic strains to the intestinal epithelium and the subsequent colonisation of the human GI-tract have been proposed as one of the pre-requisites for probiotic action. Adhesion has been advocated to provide an interaction of strains on the mucosal surface facilitating the contact with gut associated lymphoid tissue mediating local and systemic immune effects. Thus, adherent strains of PB are likely to persist longer in the intestinal tract and thus have better possibilities of showing metabolic and immunomodulatory effect (Salminen et al., 1996). Adhesion has also been reported to assist in competitive exclusion of pathogenic bacteria from intestinal epithelium (Coconnier et al., 1993). Thus, adherent probiotic strains are believed to provide better antagonism against pathogens when compared with non-adherent strains (Vesterlund et al., 2005). Adherence of PB is also important for persistence of PB in the intestine, especially small intestine, where flow rates are comparatively high (Alander et al., 1997). Feeding studies have determined that once the consumption of PB is stopped, particular probiotic strains can no longer be detected in the faeces after 1 or 2 weeks (Benno et al., 1996, Tannock et al., 2000) while, longer colonisation of the intestinal mucosa has been reported (Alander et al., 1997). Thus, good adhesion to intestinal mucosa is regarded as important. Immunomodulation has also been linked to the adhesion of PB to intestinal mucosa (Ouwehand and Salminen, 2003). Adhesion of PB to intestinal mucosa is thought to prevent the binding of some pathogenic microorganism by a mechanism known as competitive exclusion (Bernet et al., 1994, Gopal et al., 2001). Competitive exclusion is a competition between microorganisms for nutrients and binding sites as well as involves production of antimicrobial compounds thus inhibiting pathogens (Adlerberth et al., 2000). Even though no definite correlation appears to exist between *in vitro* adhesion and pathogen exclusion (Gopal et al., 2001), presence of higher concentration of PB (with high affinity to attachment receptors) than the pathogens have been demonstrated to displace adhering pathogens (Lee et al., 2000). In their research, experimentally damaged gastric mucosa showed faster healing when colonised with *Lactobacillus* species. The damaged tissue was observed to be colonised by *E. coli* when no probiotic colonisation occurred and delay in healing of the mucosal tissue was reported. The most widely employed *in vitro* models to assess the adhesion ability of PB include intestinal epithelial cells (tissue culture cells like Caco-2 and HT-29 cells) and immobilized intestinal mucus, isolated from faeces or from resected tissue. Alternatively, cells like buccal epithelial cells, urinary tract epithelial cells and vaginal epithelial cells have been suggested to be used

for examining the adhesion due to cancerous nature of tissue culture cells. The reliability of the results from *in vitro* studies and their relevance *in vivo* is still contested. Moreover, the adhesion of PB *in vitro* is influenced by several factors including bacterial concentration, incubation time, buffer composition, growth medium composition and conditions as well as host specificity (Ouwehand and Salminen, 2003).

The inhibition of pathogenic bacteria has also been credited to the ability of probiotic strains to produce antimicrobial substances. Low molecular weight metabolites (such as hydrogen peroxide, lactic and acetic acid, and other aroma compounds) and secondary metabolites produced by probiotic bacteria exhibit a wide inhibitory spectrum against a number of harmful microorganisms (Helander et al., 1997, Niku-Paavola et al., 1999).

2.1.4.2 Viability of probiotic bacteria

The health benefits from PB can be anticipated only when viable cells can survive their translocation through stomach and digestive system and subsequently colonise the gut (Kailasapathy and Chin, 2000). Moreover, the production of probiotic food has presented various technological challenges owing to the sensitive nature of PB. Some probiotic strains like *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* are sensitive to bile and acidity and are reported to get killed during the passage through the intestine (Gilliland, 1979). *L. acidophilus* and *B. bifidum* in contrast, has been reported to establish themselves among the gut flora when consumed through yoghurt (Tamime et al., 1995). The buffering capacity of the yoghurt is believed to protect the bacteria in the hostile environment of GI tract (Supriadi et al., 1994). Hence, it is a common practice to consume PB through food.

The range of functional foods with probiotics available in the market has extended over the years. For the food industry to succeed in promoting the consumption of functional probiotic products, it has to satisfy the demands of the consumer. Reports on technological aspects of microorganisms in functional foods concentrate almost exclusively on the growth or viability of probiotic strains (Knorr, 1998). The food with probiotics labelled as having “functional properties” must carry specific probiotic strains at a suitable level (10^6 - 10^7 CFU/mL) until the end of shelf-life of the product. Moreover, all probiotic foods should be safe and have good sensory properties (Saarela et al., 2000).

Before probiotic strains can be delivered to consumers, they must first be able to be manufactured under industrial conditions, and then survive and retain their functionality during storage as frozen or freeze dried cultures, and also in the food products into which they are finally formulated. The packaging material used and the storage conditions under which the products are stored are crucial for maintaining the quality of probiotic products (Saarela et al., 2000). Extensive research carried out on the viability and survivability of probiotics in the GI tract and food products has revealed that, in general, their viability greatly decreases owing to hostile environmental factors such as organic acids, hydrogen ions, molecular oxygen and antibacterial components (Gilliland and Speck, 1977, Iwana et al., 1993, Mortazavian et al., 2006, Shah et al., 1995, Sultana et al., 2000).

Hence, a stringent selection of probiotic strains for technological performance is vital. The main technological properties of probiotics (given that they should fulfill the desired biological effect and have no toxicity) include oxygen tolerance, acid tolerance, bile tolerance, heat tolerance, ability to grow in milk, ability to metabolise prebiotics, not adversely affect product quality or sensory characteristics, and be stable to commercial conditions.

2.1.5 Mechanisms of probiotic action

In order to better understand the influence of PB on the well-being of humans, the clear understanding of mechanisms involved in health-promoting functions of these organisms is vital. The intestinal epithelium and the intestinal microbiota acts as a barrier to the movement of pathogenic bacteria, antigens and other detrimental substances from the gut lumen. Normally, this barrier remains intact and supports a normal intestinal protection, but if the epithelial cells or the normal microflora are disturbed, the altered permeability facilitates the invasion of pathogens, foreign antigens and other noxious substances. In such a scenario, the use of probiotic bacteria presents a viable and safe therapy option (Salminen et al., 1996). By reinforcing the body's natural defences and keeping out harmful pathogens by competitive exclusion, probiotics make their way to grow and colonise in the intestinal wall (Anal and Singh, 2007). The figure 2.3 illustrates the main mechanisms of action of probiotics in human colon. These mechanisms can be broadly classified into three categories as discussed below.

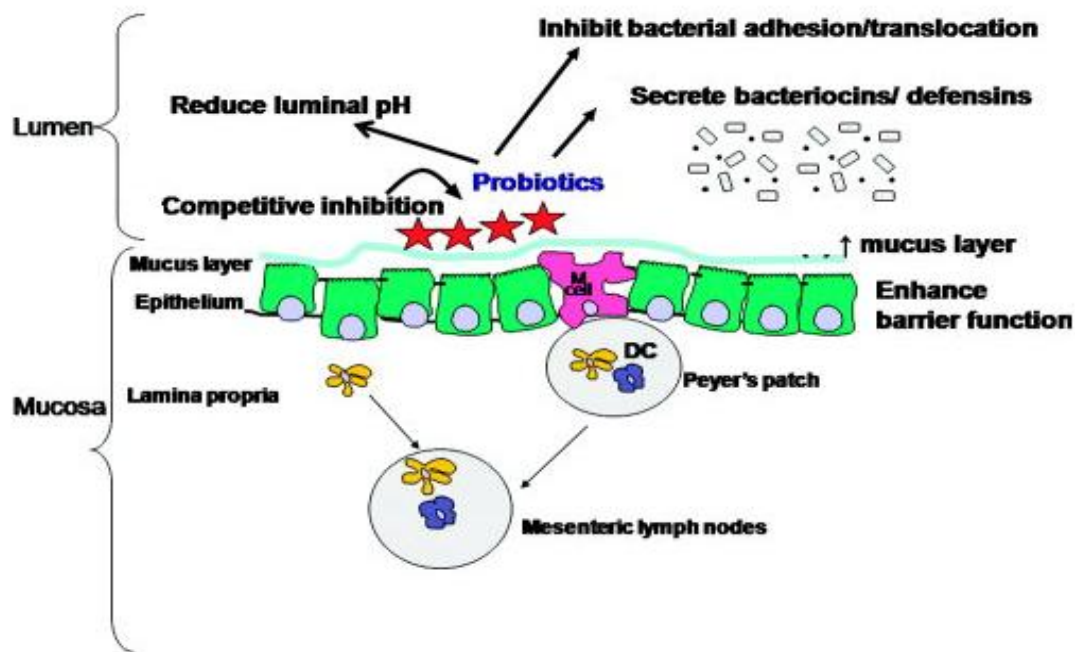


Figure 2.3. Schematic representation of mechanisms of probiotic actions on lumen and intestinal mucosa (Ng et al., 2009)

2.1.5.1 Antimicrobial effects

During the fermentation of carbohydrates in the lumen of the colon, pyruvate is converted to lactic acid and other short chain fatty acids (SCFA) such as acetic, propionic and butyric acid by the action of lactate dehydrogenase. These acids, in turn, reduce the intestinal lumen pH, creating a hostile environment for the growth of Gram-positive and Gram-negative bacteria (Cummings and Macfarlane, 1997, Kailasapathy and Chin, 2000, Mishra and Lambert, 1996, Nag, 2011, Ng et al., 2009). Lactic and acetic acids that occurs in undissociated forms in the colon function possibly by permeating the cell membrane of pathogens which interferes with the transmembrane potential and substrate transport, subsequently reducing the proliferation of undesirable microbes (Maloney, 1990). Lactic acid produced by *L. acidophilus* was demonstrated to inhibit growth of *Helicobacter pylori* in an *in vitro* study by Midolo et al. (1995). Lactic and acetic acids produced by *B. infantis* have been determined to exhibit an inhibitory effect against *E. coli* and *Clostridium perfringens* (Gibson and Wang, 1994). In addition to reducing pH, probiotics have also been reported to produce phenols, ammonia, steroid metabolites and bacterial toxins that prevent the growth of pathogenic microorganisms

(Mishra and Lambert, 1996). LAB such as *Lactobacillus* and *Bifidobacterium* produce hydrogen peroxide (H_2O_2) which acts by oxidizing thiocyanate to liberate hydrocyanic acid that kills food pathogens. The thiocyanate anion is ubiquitously present in animal tissues and secretions and is reported to be derived from the metabolism of sulphur amino acids in the diet (Fernandes et al., 1987). LAB producing H_2O_2 have been demonstrated to render a lethal effect on *Pseudomonas* spp. (Price and Lee, 1970) and *S. aureus* (Dahiya and Speck, 1968).

The production of antimicrobial compounds, termed bacteriocins, by probiotic bacteria is also likely to contribute to their beneficial activity. Bacteriocins are a heterogeneous group of anti-bacterial proteins that vary in their mode of action, spectrum of activity, molecular weight, genetic origin and biochemical properties, with bactericidal activity directed against the species that are usually closely related to the producer bacterium (Abee et al., 1995, Klaenhammer, 1988). The major classes of bacteriocins include : lantibiotics, small heat stable peptides, large heat labile proteins, and complex proteins whose activity requires the association of carbohydrate or lipid moieties (Klaenhammer, 1993). Nisin, pediocin, sakacin and reuterin (3-hydroxyl propionaldehyde) are reported to be the major bacteriocins produced by LAB.

Nisin synthesised by *Lactococcus lactis* subsp. *lactis* has been reported to exhibit a broad inhibitory spectrum against not only closely related bacteria, such as species from the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*, but also inhibit many less closely related Gram-positive bacteria, such as *L. monocytogenes*, *S. aureus*, *Bacillus cereus*, and *Clostridium botulinum* (Chen and Hoover, 2003). The antagonistic activity of nisin has been attributed to its ability to form pores in cell membranes, as well as its capacity to act on energised membrane vesicles to disrupt the proton motive force, inhibit uptake of amino acids, and cause release of accumulated amino acids (Sahl et al., 1995).

Pediocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0 is a broad spectrum bacteriocin capable of inhibiting different species of *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *Staphylococcus* spp., *B. cereus*, and *Clostridium* spp. Similarly, reuterin synthesised by *L. reuteri* is capable of inhibiting *Salmonella*, *Shigella*, *Clostridium* and *Listeria*, while, sakacin-A produced by *Lactobacillus sake* LB706 is active against different species of *Enterococcus*, *Lactobacillus*, *Pediococcus*, *L. monocytogenes*, *L. innocua* and *L. ivanovi*, but cannot inhibit *Lactococcus* and *Leuconostoc* (Chen and Hoover, 2003).

Hexose fermentation by heterofermentative lactobacilli results in the production of carbon dioxide (CO₂) (Lindgren and Dobrogosz, 1990). The anaerobic condition produced by CO₂ makes the survival of aerobic bacteria difficult. Moreover, the CO₂ produced by LAB hinders enzymatic decarboxylation, and the concentration of gas in the lipid - bilayer causes disruption of the cell membrane thus, exerting an anti-bacterial effects (Eklund, 1984). Jay (1982) noted that the synthesis of diacetyl by LAB from pyruvate inhibits the proliferation of disease - causing *Mycobacterium tuberculosis*, *Corynebacterium diphtheriae*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Pseudomonas spp.* *Salmonella spp.*, *S. aureus* and *Streptococcus spp.* Hugenholtz et al. (2000) reported that diacetyl reacts with arginine-binding protein, thus preventing the utilisation of arginine by Gram-negative bacteria, leading to the inhibition of growth.

2.1.5.2 Enhancement of barrier function

Apart from inhibiting the growth of potential pathogens, probiotics possess the ability to influence mucosal cell-cell interactions and cellular “stability” by the enhancement of intestinal barrier function through the modulation of cytoskeletal and tight junctional protein phosphorylation (Ng et al., 2009). The intestinal barrier function is maintained by several interrelated systems including mucus secretion, chloride and water secretion, and binding together of epithelial cells at their apical junctions by tight junction proteins. The disruption of epithelial barrier function is seen in several conditions, including inflammatory bowel diseases (Irvine and Marshall, 2000, Schmitz et al., 1999), enteric infections (Sakaguchi et al., 2002) and Type 1 diabetes (Watts et al., 2005). The enhancement of mucosal barrier function may be an important mechanism by which probiotic bacteria benefit the host in such diseases (Meddings, 2008).

The enhancement of barrier function by PB has been examined both, in *in-vitro* models and *in-vivo* in the whole animal. The probiotic mixture VSL#3 normalised colon physiologic functions and barrier integrity and also enhanced resistance to *Salmonella* invasion in mice (Madsen et al., 2001). The increased barrier integrity in response to PB has been observed in healthy animals and in animal models of colitis. In a study by Garcia-Lafuente et al. (2001), *L. brevis* enhanced barrier function as assessed by permeability to mannitol in excluded colonic

loop in rats. In interleukin-10-deficient mice with chronic colitis, *Lactobacillus* improved barrier function *in vivo* (Madsen et al., 1999). The manipulation of human intestinal microflora using PB offers a good alternative to antibiotic therapy, and hence, food carrying these health-promoting microorganisms is in great demand.

2.1.5.3 Immunomodulation

PB are claimed to modulate immune response to harmful antigens through production of B lymphocytes and antibodies (Ng et al., 2009). Kaila et al. (1992) observed improvement in non-specific humoral immune response in children with acute gastroenteritis when administered with *L. rhamnosus* GG. This response was reflected by the rise in IgG, IgA and IgM immunoglobulin secretions from circulating lymphocytes. *B. bifidum* has been demonstrated to improve antibody response to ovalbumin while, yoghurts containing *L. acidophilus*, *B. bifidum* and *B. infantis* enhanced the IgA response to cholera toxin in mice (Tejada-Simon et al., 1999). The vaccination trials have also confirmed the enhanced antibody production using PB. *L. casei* was shown to increase immunogenicity of rotavirus vaccination in children (Isolauri et al., 1995) while, *L. rhamnosus* GG and *Salmonella* vaccination in combination has been determined to enhance *Salmonella*-specific IgA levels (Fang et al., 2000).

L. casei has also been reported to improve natural killer (NK) cell activity *in vivo* and *in vitro* in humans in conjunction with the production of interleukin IL-12 (Takeda et al., 2006). *L. casei* in conjunction with dextran has been shown to improve humoral immune response by significantly elevating the NK cell activities in mice by (Ogawa et al., 2005). The following study by Ogawa et al. (2006) demonstrated the efficient induction of NK cell activities and elevation in IL-12 production in human volunteers via oral administration of *L. casei* in conjunction with dextran.

2.1.6 Health claims and safety concerns associated with probiotic bacteria

2.1.6.1 Health benefits of probiotic bacteria

There is a huge pool of research publications regarding the array of probiotic applications in human clinical trials. This subject is not within the scope of the current review, but a short glimpse of the important and common studies is discussed below, particularly relating to *Lactobacillus* and *Bifidobacterium* species.

Clinical symptoms claimed to be treated or having the potential to be treated with probiotics include decrease in the incidence and duration of diarrhoea, irritable bowel syndrome, inflammatory bowel disease, food allergies and atopic eczema. Probiotics have also been reported to reduce lactose intolerance, hypertension, hepatic diseases and to enhance the immune system (Kailasapathy and Chin, 2000, Salminen et al., 1998b). Some health benefits of PB have been well determined, while other benefits have displayed positive results in animal models and need further investigation in humans. Health benefits conferred by PB are reported to be strain specific and no strain will impart all the proposed benefits (Shah, 2007).

Oral administration of *Lactobacillus* and *Bifidobacterium* has been established to restore the balance of intestinal microbiota by suppression of pathogens including *Escherichia coli*, *Salmonella typhimurium*, *S. aureus* and *Clostridium perfringens* (Shah, 2007). Dairy products enhanced with *B. breve* have been shown to prevent diarrhoeal disease associated with *Campylobacter* in Japanese children (Tojo et al., 1987). In an *in-vitro* study, a fermented milk product containing *Lactobacillus* has been determined to prevent and treat diarrhoea induced by *Salmonella* or *Shigella* (Alm, 1983). This effect has been attributed to the production of bacteriocins by the *Lactobacillus* strain (Barefoot and Klaenhammer, 1983). When cultured milk carrying *L. acidophilus*, *S. thermophilus* and *B. longum* was administered to senile patients with diarrhoea, it proved to be effective in reducing stool frequency, and their condition relating to diarrhoea was improved. Moreover, the number of Bifidobacteria in their faeces increased (Takiguchi et al., 1985). In a similar study, *Lactobacillus* GG in yoghurt has been used to treat antibiotic-associated diarrhoea. Volunteers who received the PB with erythromycin showed less diarrhoea than those who consumed pasteurised yoghurt as a control (Siitonen et al., 1990). Administration of *Lactobacillus* GG has also been demonstrated to

terminate episodes of relapsing diarrhoea caused by a toxin-producing strain of *Clostridium difficile* (Gorbach et al., 1987).

Use of probiotics to sooth the symptoms of lactose intolerance is probably the most widely accepted health benefit. Lactose intolerance or mal-absorption occurs because of the inability to digest lactose adequately owing to the absence of the enzyme β -galactosidase in the human intestine that normally hydrolyse the principal component of milk into glucose and galactose. Lactose malabsorption can often lead to abdominal discomfort after consumption of fresh, unfermented milk or milk products, owing to the generation of hydrogen gas by microbial action on undigested lactose in the gut (Shah, 1993, Shah et al., 1992). The efficacy of yoghurt compared with milk have been well documented in counteracting lactose intolerance (Vrese et al., 2001). The traditional yoghurt cultures, *L. delbrueckii* subspecies *bulgaricus* and *Streptococcus thermophilus* contain a considerable amount of β -galactosidase and hence, yoghurt and probiotic yoghurt appear to be a good remedy for lactose malabsorbers (Shah, 2000, Shah, 2007). Lysis of bacteria by bile juices upon ingestion is thought to release the enzyme and hydrolyse lactose (Vesa et al., 2000). Hence, milk containing or fermented with *L. acidophilus*, that survives the intestinal transit, is actually less effective than milk containing or fermented with *S. thermophilus* that are killed upon ingestion (Lin et al., 1991).

Probiotics have also been reported to present anticarcinogenic and antimutagenic activity. PB reduces the amount of carcinogens generated in the intestine by preventing the growth of bacteria that convert procarcinogens into carcinogens (Hata et al., 1996, Lee et al., 2004). The compounds produced during the growth of LAB to a substantial level have been credited with anticancer and antimutagenic action (Sophorn, 2010). In addition to suppressing procarcinogen convertors, stimulation of immune system and the reduction of intestinal pH has been accounted for anticarcinogenic activity of *L. acidophilus* (Gilliland, 1989). Through dietary supplementation with *L. acidophilus*, De Santis et al. (2000) observed reduction in the total number of colon cancer in rats. *B. longum* has also been determined to cease the incidence of colon, liver, intestinal and mammary tumors in rats (De Santis et al., 2000). *L. acidophilus* strain DDS1 has been highlighted to generate pronounced antitumor activity (Shahani et al., 1983). In regards to antimutagenic activity, PB functions by binding to mutagens, thereby reducing faecal enzymatic activities that are involved in the stimulation of mutagens (Goldin and Gorbach, 1984, Orrhage et al., 1994). Lankaputhra and Shah (1998) demonstrated the marked efficacy of butyric acid produced by *L. acidophilus* and

Bifidobacterium strains to exhibit a wide spectrum antimutagenic action against all mutagens and promutagens investigated. Moreover, the live cultures in their study displayed more effective antimutagenic activities when compared with those of killed cells. This reflects the importance of administration of live PB and maintaining their viability in the intestine to effectively weaken mutagens.

The consumption of fermented milk carrying a high number of PB (10^9 CFU/g) by hypercholesterolaemic patients has been reported to lower cholesterol from 3.0 to 1.5 g/L (Shah, 2007), which has been correlated with substantial reduction in the risk of cardiovascular disease (Gilliland, 1989). Some *L. acidophilus* and *Bifidobacterium* strains have been shown to lower the cholesterol level. PB are reported to deconjugate bile salts and the cholesterol co-precipitates with deconjugated bile salts as the pH declines as a result of lactic acid production by LAB. Deconjugated bile acid is less absorbed than its conjugated counterpart, ultimately resulting in lower absorption of cholesterol. Uptake of cholesterol by *L. acidophilus* during their growth has also been reported, making it unavailable for absorption into the blood stream (Klaver and Van der Meer, 1993). Lactobacilli also proved effective in treatment of functional intestinal disorders, including irritable bowel syndrome (IBS) (Sophorn, 2010). A significant improvement in patients with IBS symptoms was noted when the patients were administered with *L. plantarum* 299V suspension (Niedzielin and Kordecki, 2001). In a study by Jonsson et al. (1983), *L. plantarum* exerted a positive effect on several, well characterised symptoms of IBS, which was credited to the ability of this bacterium to catabolise arginine and generate nitric oxide. Nitric oxide has been reported to exert a positive effect on the motility of the large and small intestines, resulting in the improvement of the splanchnic circulation and immunological processes (Wright et al., 1992).

The effective influence of PB on the immune response has been extensively reviewed. The ability of PB to improve the immune response, either directly or indirectly, is reflected by the evidence from *in vitro* and animal studies as well as in humans (Perdigon et al., 1995). Immune response stimulation by PB has been thought to be conferred through activation of macrophages, raising the levels of cytokines, increasing the activity of natural killer cells and/or escalating the levels of immunoglobulins (Ig) (Ouwehand et al., 2002, Shah, 2007). Higher levels of total and anti-poliovirus IgA have been reported in Japanese children consuming probiotic formula carrying *B. lactis* (Fukushima et al., 1998). The oral

administration of Lactobacilli has also been reported to induce stimulation of macrophages and lymphocytes in mice (Perdigon et al., 1986).

The positive effect of probiotics beyond the gut has also been demonstrated. The consumption of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have been determined to present a positive effect on vaginal health (Anukam et al., 2006). *L. paracasei* ST11 have been reported to enhance the recovery of skin immune homeostasis (Peguet-Navarro et al., 2008). A few studies have also demonstrated the efficacy of probiotic therapy in modulating mood (Benton et al., 2006) and stress-induced gastrointestinal symptoms in human subjects (Diop et al., 2008).

2.1.6.2 Safety guidelines for the evaluation of probiotic bacteria

Although a long history of safe application of LAB especially in food-fermentation has been exclusively documented, the exposure of human body to novel probiotic strains could be risky (Salminen et al., 1998c). The outcomes of a number of clinical trials have raised the concern for safe application of probiotics. Robert Havenaar and Spanhaak (1994) expressed that the risk of septicaemia, endocarditis and local infections and side effects like excessive inflammatory response cannot be overlooked. Moreover, the use of genetically modified PB could lead to the emergence of antibiotic resistant pathogens. Egervarn et al. (2009) identified antibiotic-resistant genes in *L. reuteri* and *L. plantarum* which suggests the potential risk of transferring antibiotic resistant genes to the host. The consumption of *L. rhamnosus* have been associated with bacteremia in patients by Rautio et al. (1999) and this strain has been reported to be indistinguishable from *L. rhamnosus* strain GG. *Lactobacillus*-associated bacteraemia has also been reported in immunocompromised and immunocompetent patients (Salminen et al., 2004). This has created complexity among physicians to prescribe probiotics for patients with immunocompromised patients and those undergoing surgery (Reid and Hammond, 2005) since, Olah et al. (2002) noted improvement in health of patients suffering from acute pancreatitis as well as in patients that underwent abdominal surgery, by consumption of probiotics. Antony (2000) expressed that despite the safe history of probiotics in majority of human clinical studies, it is important for the physicians to supervise their use in high-risk individuals.

The safety of probiotic strains is of prime importance and guidelines for the safety assessment have been well documented in many publications (Boyer and Liu, 2004, Salminen et al., 1996, Salminen et al., 1999, Salminen et al., 1998a, Salminen et al., 1998b, Salminen et al., 1998c). They have suggested three approaches to assess the safety of probiotic microorganisms: studies on the intrinsic properties of the strain, the pharmacokinetics of the strain (survival, activity in the intestine, dose–response relationships, faecal and mucosal recovery) and studies searching for interactions between the strain and the host. The selection criterion for probiotics is illustrated in figure 2.4. The significance of human origin has been debated recently, but the most current successful strains are indicated to be of human origin. Saarela et al. (2000) claims that a probiotic strain can function better in an environment (e.g. human GI-tract) similar to the one from which it was originally isolated, and hence, probiotic strains of human origin tend to be safe and should be recommended for use by humans.

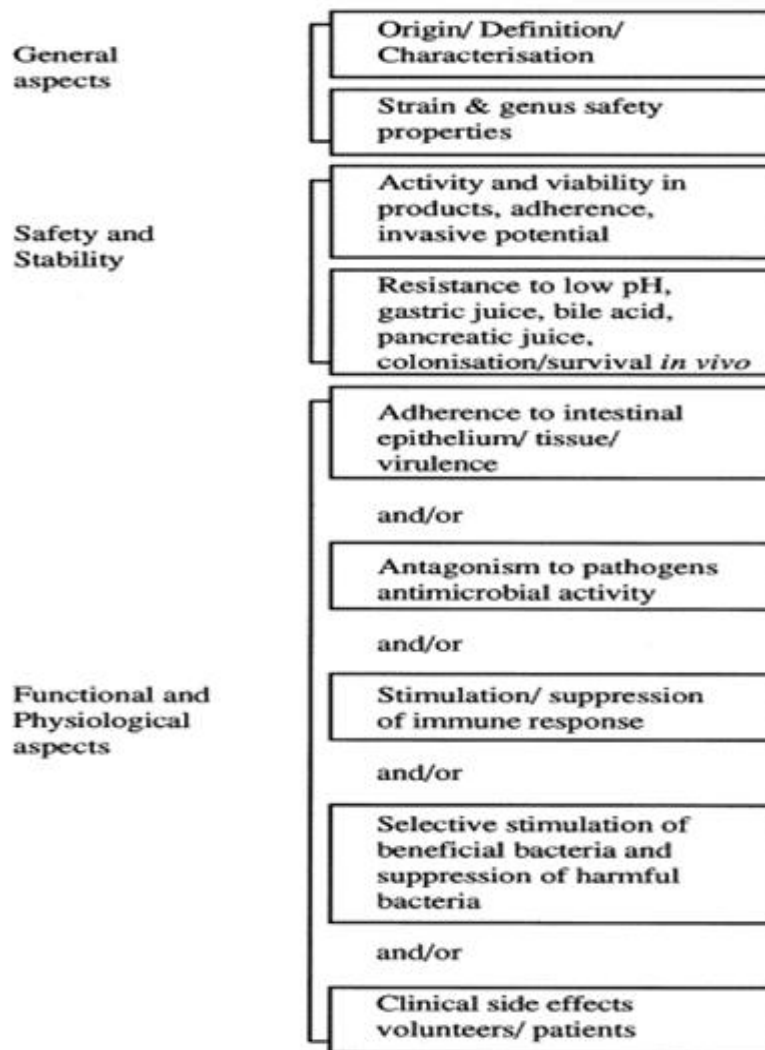


Figure 2.4. Selection criteria for probiotics (Salminen et al., 1998c)

Even though no general guidelines for the safety testing of probiotics exists, many countries including the European Community have been reported to be in the process of developing more detailed guidelines with respect to regulations for novel and functional foods and related probiotic preparations (Donohue and Salminen, 1996). The information regarding the survival of the probiotics in the GI-tract, their translocation and colonisation attributes, aids in the evaluation of their potential positive effects as well as side-effects (Salminen et al., 1998c). In the European Union, the Novel Food Regulation (258/97/EC) regulates the probiotics (Ezendam and Loveren, 2006). This regulation is reported to apply to only those strains that were not used before 1997 and concerns novel foods or food ingredients. According to the regulations in Denmark, the manufacturer prior to the employment of new probiotic strain is required to notify the relevant authority. In France, a

premarket approval system for novel strains is being considered and the proposed recommendations were published by Agence Francaise de Se´curite´ Sanitaire des Aliments (Ezendam and Loveren, 2006).

In the United States, the Food and Drug Administration (FDA) regulates the probiotic food and the approval is based on the safety and efficacy data and/or if it can be considered “generally recognised as safe” (GRAS). The GRAS status is given to a particular probiotic strain only when it has a safe history of usage in food dating prior to 1958 or has been determined as safe by experts. Food and Agricultural Organisation of the United Nations (FAO) and World Health Organisation (WHO) has designed the scheme for evaluation of probiotics for use in food items as shown in figure 2.5. These guidelines can be applied to evaluate the safety of novel probiotic strains to be used in food products (FAO/WHO, 2002).

The range of foods incorporating probiotics is extending at a very fast pace from dairy foods to infant formulae, baby foods, fruit juice-based products, cereal-based products and pharmaceuticals. New and more specific strains of probiotic microorganisms are being investigated and explored. Prior to incorporation of novel strains into food, each strain must be carefully and thoroughly examined and tested for the safety and efficacy of their proposed use.

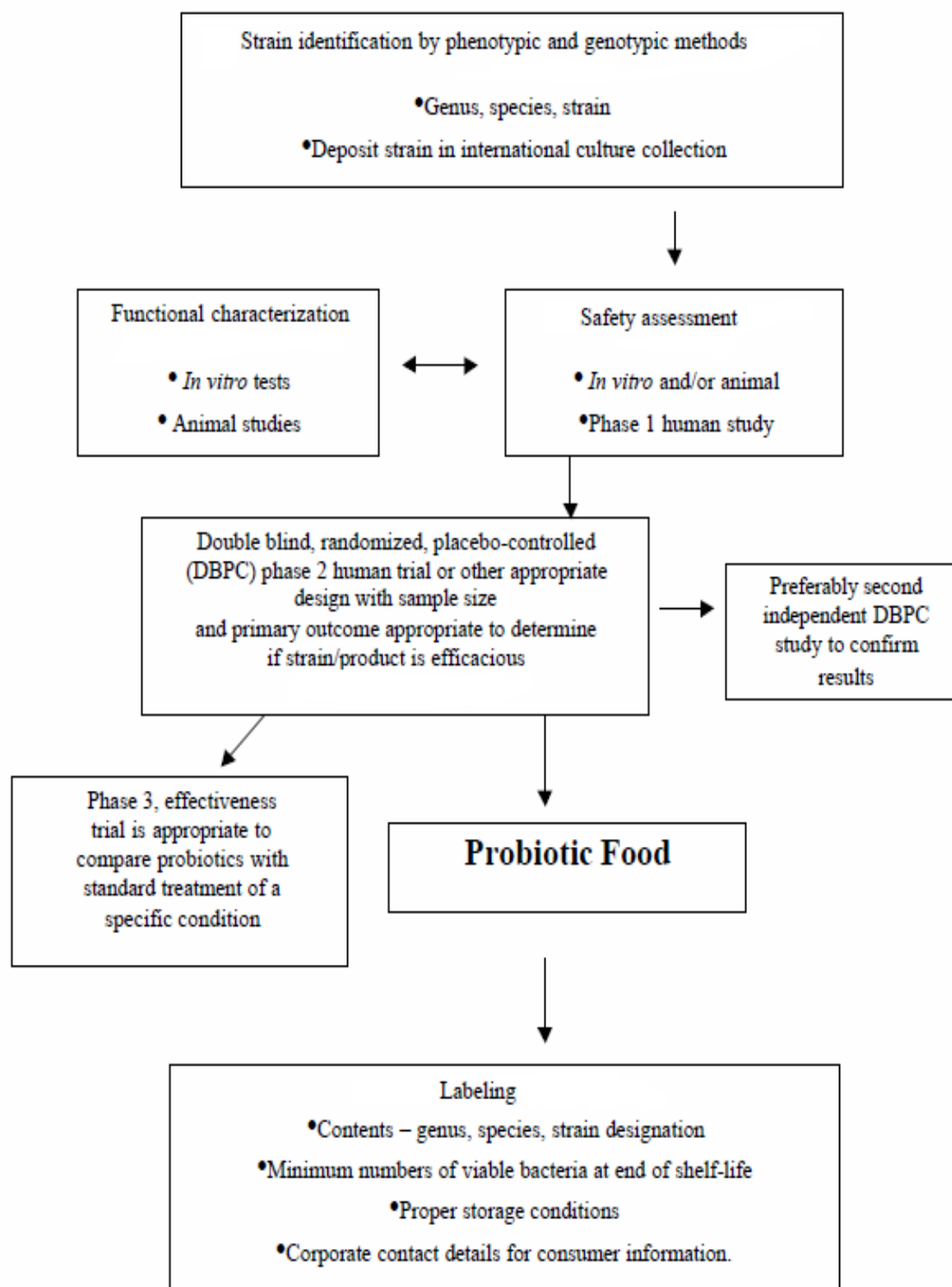


Figure 2.5. The guidelines for the evaluation of probiotics for use in food (Whitehead et al., 1980)

2.1.7 Probiotic-enhanced foods and associated challenges during their development

The increased awareness among consumers on the role of probiotic food in healthy diet has boosted the production of a huge variety of foods carrying PB. Dairy products are the key sector among the probiotic foods, with yoghurts, kefir and cultured drinks representing major categories (Granato et al., 2010b). Probiotic cheese, ice cream, nutritional bars, breakfast cereals and infant formula are also gradually gaining popularity (Cruz et al., 2009, Cruz et al., 2007, Gardiner et al., 1999). In Europe probiotic food market is reported to be >1.4 billion euros of which yoghurt and desserts account for a sale of about 1 billion euros, and the rest of the market is dominated by probiotic milks. Annual growth of the market is estimated at 7-8% over the next 5 years (Saxelin, 2008). The most popular format in Europe is reported to be the “daily-dose” drinks (a single serving of 65-125 mL, which is supposed to carry an effective dose of PB), like the drinks introduced by the Japanese company Yakult. The non-dairy PB products, like non-alcoholic beverages produced with cereals as the main raw material are also gaining importance with the continuing trend to vegetarianism, as well as the problems of lactose intolerance and cholesterol content of dairy products (Granato et al., 2010b, Prado et al., 2008). This review will focus only on dairy products as a probiotic carrier.

PB needs to be viable in the food at a minimum concentration of 10^6 CFU/g of product in to be able to get the health benefits from them (Kailasapathy and Chin, 2000, Shah, 2007). Though many products claim their 'probiotic-status' in the market, and despite the importance of viability, investigatory studies have shown low numbers of PB ($< 10^6$ CFU/g) in these health-claiming food products (Gueimonde et al., 2004, Shah, 2000, Shah et al., 2000). Enumeration for viable *L. acidophilus* and *B. bifidum* in five commercial Australian yoghurts by Shah et al. (1995) revealed that only three of five products carried viable 10^7 - 10^8 *L. acidophilus* cells/g while two samples contained $\leq 10^5$ /g of these cells. In case of *B. bifidum* viable count in two of the tested yoghurts was found to be 10^6 - 10^7 *B. bifidum* cells/g while, $\leq 10^3$ cells/g in the remaining three samples. Moreover, a consistent drop in the viability of both the probiotic bacteria was reported in all the products during five 5 weeks storage period. In a similar investigation, Iwana et al. (1993) detected that three of eight yoghurts in the U. K. tested negative for *Bifidobacterium*. The remaining five yoghurts contained viable count of 10^4 - 10^7 viable cells/mL. The viable counts of *Lactobacillus* spp. in commercial fermented milks was determined to be $>10^5$ CFU/mL while, the numbers of *Bifidobacterium* spp.

dropped below this level in two products in yet another study (Gueimonde et al., 2004). Hence, it is inaccurate to market products as “health-promoting” unless they carry the required minimum level of viable probiotic cells until the expiry date. It is still arguable whether such products can deliver the claimed health benefits (Shah, 2007). Clements et al. (1983) claim that non-viable *Lactobacillus* cells hold the ability to promote health. In their experiment, one out of the two lyophilized *Lactobacillus* preparation tested lowered the course of diarrhoea, while the second batch proved ineffective. Such discrepancies could arise due to inadequate lyophilisation and requires further investigation. Hence, it cannot be completely agreed that non-viable cells in their study were effective in lowering diarrhea.

Dairy products like yoghurt is a well appreciated carrier of probiotic bacteria. Several investigatory studies have been conducted involving examination of commercial yoghurts in the market places of Australia and Europe for the presence of *L. acidophilus* and *Bifidobacterium* and have reported variability in the concentration of *L. acidophilus* in the products and low levels of *Bifidobacterium* (Huys et al., 2006, Iwana et al., 1993, Masco et al., 2005, Micanel et al., 1997, Temmerman et al., 2003, Tharmaraj and Shah, 2004, Varnam and Sutherland, 1994, Vinderola et al., 2000). Several factors have been blamed for the decline in PB cells in yoghurt including acidity, oxidative stress in the product, oxygen permeation through the package, sensitivity of PB to antimicrobial substances produced by starter cultures and lack of nutrients in the milk (Dave and Shah, 1997a, Shah et al., 2000, Tamime et al., 1995, Vinderola et al., 2000). Numerous factors responsible for hindering the viability and activity of PB during culture preparation, production and delivery to the gut are summarized in table 2.2.

Table 2.2. Factors affecting the viability and activity of probiotic bacteria (Vasiljevic and Shah, 2008)

Processing step	Stress vector
Production of probiotic preparations	Presence of organic acids during cultivation Concentration—high osmotic pressure, low water activity, higher concentration of particular ions Temperature—freezing, vacuum and spray drying Drying Prolonged storage—oxygen exposure, temperature fluctuation
Production of a probiotic containing product	Nutrient depletion Strain antagonism Increased acidity Positive redox potential (presence of oxygen) Presence of antimicrobial compounds, i.e., hydrogen peroxide and bacteriocins Storage temperature
Gastrointestinal transit	Gastric acid and juices Bile salts Microbial antagonism

A wide range of other dairy products has been explored as a potential vehicle for the delivery of PB including ice cream (Akin et al., 2007, Hekmat and McMahon, 1992), frozen fermented dairy desserts (Magarinos et al., 2008), chocolate mousse (Aragon-Alegro et al., 2007) and freeze-dried yoghurts (Capela et al., 2006, Rybka and Kailasapathy, 1995). However, cell injury due to freezing and freeze-drying in these innovative products affected their efficacy of delivering required the level of PB (Cruz et al., 2009, Shah, 2007).

Tharmaraj and Shah (2004) suggested the use of cheese-dip as a probiotic carrier because of its stable pH, the buffering capacity of the ingredients employed and the incorporation of prebiotics. Cheddar cheese has also been examined as a potential vehicle of PB. Owing to reduced acidity, more solid consistency, high buffering capacity and high fat content, cheese has advantages as a carrier over fresh fermented products (Stanton et al., 1998). Moreover, the texture of cheddar cheese has been reported to provide protection to PB during gastric transit (Gardiner et al., 1999). However, cheddar cheese needs a considerably long time to ripen and hence, can employ only specific probiotic strains that are capable of sustaining viability throughout the ripening and shelf life (Ong et al., 2006).

Although a growing number of revelations claim the health benefits of consuming probiotics, no agreed parameters for selection standards exists for the classification of bacteria as probiotic (Hasler, 2002). The commonly used criteria as enlisted by Kailaspathy and Chin (2000) include "human origin, tolerance to intestinal conditions, ability to bind and colonize intestinal mucosa, synthesis of antimicrobials, demonstrable viability, proficiency and safety". They further expressed the view that the following few elements cannot be ignored during the screening process: the ability of organisms to maintain the desired characteristics during commercial production and storage, and the need for *in vitro* studies and clinical trials. The endurance of PB is also reported to be determined by several other food-associated attributes including pH, concentration of metabolites, storage and culturing conditions as well as the strains of PB (Rybka and Kailasapathy, 1995, Shah, 2000, Shah et al., 2000).

It is evident that the food matrix plays an important role in influencing the viability and activity of PB and hence, a careful selection of strains based on the application and function is necessary. Also, huge efforts are being put to sustain the viability and activity of PB in the currently available food products by manipulation of manufacturing conditions (Kailasapathy and Chin, 2000).

2.1.8 Methods to increase survival of probiotic bacteria in food

Several strategies have been proposed to increase the survival of PB in foods. Varnam and Sutherland (1994) suggested ceasing fermentation at higher pH in view of the greater survival of *Bifidobacteria* at pH > 5, while Kailaspathy and Chin (2000) recommended reducing the incubation temperature to 37 °C for optimal growth of *Bifidobacteria*. The application of heat-shock to yoghurt proved effective in maintaining the desired acidity during storage by reducing the acid over-production (Marshall, 1992). Sakai et al. (1987) proposed storage temperatures of < 3- 4 °C for the efficient proliferation of PB in yoghurt. Post-acidification in yoghurts responsible for declining viability of PB can be prevented by the application of hydrostatic pressure (200-300 MPa for 10 min) to yoghurt (Kailasapathy and Chin, 2000, Tanaka and Hatanaka, 1992).

Microencapsulation technology has been widely employed and has proved successful in supporting the survival of PB against human intestinal juices as well as in maintaining the viability in dairy products (Anal and Singh, 2007, Kailasapathy, 2002, Krasaekoopt et al., 2006). Microencapsulation is “a process wherein the cells are retained within an encapsulating membrane to prevent cell injury or cell loss” (Krasaekoopt et al., 2006). Several investigations have reported additional advantages of microencapsulation of PB, including protection of bacteria inside the beads from bacteriophages (Steenenson et al., 1987), improved survival during freeze drying and freezing (Kearney et al., 1990, Sheu and Marshall, 1993) and pronounced stability during storage (Homayouni et al., 2008, Kim et al., 1988). Various supporting materials including alginate (Mandal et al., 2005, Sultana et al., 2000), carrageenan, locust bean gum (Arnaud and Lacroix, 1991, Audet et al., 1988) and gelatin (Hyndman et al., 1993) have been successfully utilised to microencapsulate probiotic bacteria.

The survival of microencapsulated *L. acidophilus* 547, *B. Bifidum* ATCC 1994 and *L. casei* 01 in alginate beads was found to be higher by 1 Log in yoghurt stored at 4 °C as compared with the control in a study by Krasaekoopt et al. (2006). Previously, Krasaekoopt et al. (2004), demonstrated the effectiveness of microencapsulated PB in alginate beads to provide protection against simulated digestive juice and bile salts. The results substantiate those observed by Annan et al. (2007) and Ding and Shah (2007), who suggested the application of microencapsulation for providing protection to PB against hostile environmental stresses.

Freeze and spray drying of PB is commonly employed to produce probiotic cultures in dried forms for food application (Anal and Singh, 2007). A number of studies are available reporting spray-drying of various strains of *Lactobacillus* and *Bifidobacterium* (Corcoran et al., 2004, Desmond et al., 2002, Gardiner et al., 2000, O'Riordan et al., 2001). Spray dried powders carrying a large number of PB offer a convenient way for storage and transportation of PB for food application. Despite being an economical process for industrial scale production of probiotic cultures, it has the major drawback of causing cell injury and death. These deleterious effects have been associated with the effect of high temperature, drying and dehydration (Ross et al., 2005). Moreover, the loss of probiotic activity after few weeks of storage at room temperature has been reported (Anal and Singh, 2007). The incorporation of protectants in media prior to drying has been employed by many workers to counteract this

problem. Additions of trehalose (Conrad et al., 2000, De Castro et al., 2000), non-fat milk solids (Selmer-Olsen et al., 1999) and growth promoting factors including prebiotics (Capela et al., 2006, Desmond et al., 2002) have been explored to enhance viability during drying, storage and/or gastric transit.

The co-encapsulation of PB with prebiotics such as Hi-maizeTM, Raftiline[®] and Raftilose[®] have been demonstrated to significantly improve the survival of two *L. acidophilus* strains in yoghurt as well as during probiotic exposure to simulated gastric conditions (Iyer and Kailasapathy, 2005). In a similar study by Capela et al. (2006), the use of cryoprotectant UnipeptineTM RS 150 enhanced the viability of *L. casei* by 7% in yoghurt during the 4 weeks of refrigerated storage, while prebiotic Raftilose[®]P95 improved the viability of combined selected PB by 1.42 Log in yoghurt during storage. The fortification of milk-based medium carrying PB with gum acacia, a soluble fibre, before spray drying has been evaluated to provide enhanced survival of during powder storage (Desmond et al., 2002).

The capability of prebiotics to selectively enhance colonic implantation of probiotic organisms has presented an opportunity to develop functional products by exploiting synergism between them (Shah, 2007). Prebiotics are defined as “non-digestible food that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). The products that incorporate both prebiotics and probiotics are referred to as ‘synbiotics’ that exploit this synergy to produce health-promoting functional food ingredients (Shah, 2007). Prebiotics like fructo-oligosaccharides and inulin have been reportedly shown to selectively stimulate *Bifidobacterium* and *Lactobacillus* spp (Schrezenmeir and de Vrese, 2001). The ability of certain phytochemicals, especially PPs, to inhibit pathogenic bacteria (Su et al., 2008) and stimulate the proliferation and adhesion of colonic microbes (Parkar et al., 2008) have generated a path to utilise these bioactive compounds to formulate novel functional foods. These products have the potential to exploit the synergy between PB and PPs to influence their functional attributes, as well as impart health benefits to the consumers. The next section of the review focuses on the polyphenols from apples.

2.3 Apples – the reservoir of polyphenols

2.3.1 Apples as a natural antioxidant source

Fruits contain essential vitamins, minerals, fibre and other bioactive compounds and hence, diet rich in such bioactives has been long been considered to help reduce the risk of chronic diseases and aid in weight management (Blanck et al., 2008). Bioactive components with positive health benefiting properties in foods may come from plant, animal or microbial sources and a large proportion of these components have been reported to be derived from plants (Sun-Waterhouse, 2011). Fruits including berries, kiwifruit and apples are rich source of antioxidants that help protect mammalian cells from oxidative damage (Kaur and Kapoor, 2001). The compounds that represent the antioxidant bioactives primarily include vitamin C and PPs such as anthocyanins, phenolic acids, flavanols, flavonols and tannins (Szajdek and Borowska, 2008). Owing to the high concentration and qualitative diversity of these antioxidants in berries like bilberry, blackberry, blackcurrant, blueberry, chokeberry, cranberry, grape, raspberry and strawberry these fruits are commonly referred to as natural functional foods (Szajdek and Borowska, 2008). Most of the work involving antioxidant potential of fruits is limited to berry fruits (Kaur and Kapoor, 2001) which are available seasonally (Ben-David et al., 1997). In contrast, fruits like apple are a rich source of antioxidants (Boyer and Liu, 2004) and are available throughout the year.

Apples are not only high in antioxidants but they are commonly consumed around the world (Boyer and Liu, 2004). Apples have been determined to possess the second highest antioxidant activity after cranberry compared with other commonly consumed fruits in the United States (Sun et al., 2002). The apples in their study scored second for total concentration of polyphenols and the apples had the highest portion of free phenolics in comparison with other fruits. This indicated that these bioactives are not bound to other compounds in apples, and the phenolics may be readily available for absorption in the blood. Hence, apples can be regarded as a vital source of antioxidants that when consumed may provide various health benefits.

Apples are regarded as a healthy fruit, as they are packed with wide spectrum of nutrients (see table 2.3) and the health promoting and disease preventing effects are evident from a number of epidemiological studies (Boyer and Liu, 2004). Apples belong to the rose

family Rosaceae and genus *Malus*. Apples have been reported to originate in Europe and mid-Asia and are now grown extensively around the world (Lam et al., 2008). The significance of the proverb “An apple a day keeps the doctor away” is reflected by the association of several health-promoting effects of the fruit and the consumption of apples by many cultures over the years. The consumption of apples has been inversely associated with cancer, cardiovascular diseases, asthma, diabetes, hypercholesterolemia and weight loss (Lam et al., 2008). Much of these protective effects of apples have been linked to phytochemicals, such as PPs (Liu, 2003, Wolfe et al., 2003). Apples are a rich source of carotenoids and PPs that include flavonoids, isoflavonoids and phenolic acids (Boyer and Liu, 2004).

The antioxidant activity of PPs has been credited for scavenging the reactive oxygen species (ROS) and thus reducing the damage to mammalian cells. ROS that includes free radicals (such as superoxide anion, hydroxyl radicals) and non-free radicals (like hydrogen peroxide and singlet oxygen) are various forms of activated oxygen. These ROS are reported to participate in cellular injury and aging process. ROS are continuously generated during normal physiological events but are reported to be eliminated by antioxidant defence mechanism (Gulcin, 2006). Under pathological conditions, ROS is overproduced due to the imbalance between production of ROS and inactivation of ROS by the antioxidant system in organisms. This results in oxidative stress and damages the mammalian cells. PP antioxidants are well known to reduce the actions of ROS. PPs function by trapping the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes (Gulcin, 2006, Rao et al., 1996).



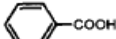
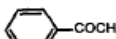
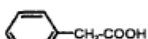


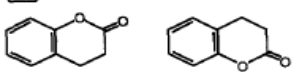
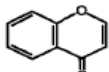
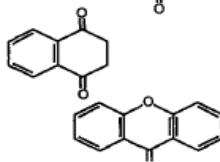
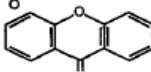
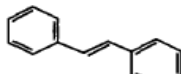
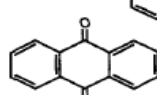
Table 2.3. Nutrient composition of raw apple with skin (amount in 100g of edible portion) (after ARS, 2011)

Nutrients	Units
Energy	52 Kcal
Protein	0.26 g
Total Fat	0.17 g
Dietary Fiber	2.40g g
Carbohydrates	13.81 g
Total Sugars	10.39 g
Vitamin A	54 IU
Vitamin C	4.6 mg
Vitamin E	0.18 mg
Vitamin K	2.2 µg
Folates	3 µg
Niacin	0.091 mg
Riboflavin	0.026 mg
Sodium	1mg
Potassium	107 mg
Calcium	6 mg
Iron	0.12 mg
Magnesium	5 mg
Phosphorus	11 mg
Zinc	0.04 mg
β-carotene	27 µg

2.3.2 Apple polyphenols

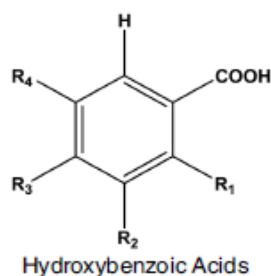
Apples contain a large concentration of PPs depending on the cultivar, harvest and storage, as well as on the processing of apples (Boyer and Liu, 2004). PPs are “compounds possessing more than one aromatic rings with one or more hydroxyl groups and are usually classified as phenolic acids, flavonoids, stilbenes, coumarins and tannins” (Liu, 2004). Bravo (1998) reported that according to Harborne (1989), PPs can be categorised into at least ten different classes, depending on their basic chemical structure as shown in table 2.4.

Table 2.4. Major classes of polyphenol compounds (Bravo, 1998)

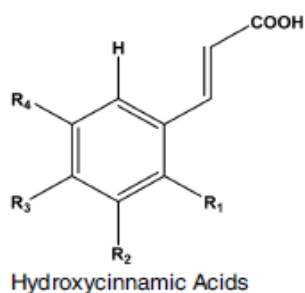
Class	Basic Skeleton	Basic Structure
Simple phenols	C ₆	
Benzoquinones	C ₆ *	
Phenolic acids	C ₆ -C ₁	
Acetophenones	C ₆ -C ₂	
Phenylacetic acids	C ₆ -C ₂	
Hydroxycinnamic acids	C ₆ -C ₃	
Phenylpropenes	C ₆ -C ₃	
Coumarins, isocoumarins	C ₆ -C ₃	
Chromones	C ₆ -C ₃	
Naftoquinones	C ₆ -C ₄	
Xanthenes	C ₆ -C ₁ -C ₆	
Stilbenes	C ₆ -C ₂ -C ₆	
Anthraquinones	C ₆ -C ₂ -C ₆	
Flavonoids	C ₆ -C ₃ -C ₆	
Lignans, neolignans	(C ₆ -C ₃) ₂	
Lignins	(C ₆ -C ₃) _n	

Phenolic acids can be divided into two groups (hydroxybenzoic and hydroxycinnamic acids) depending on the numbers and positions of the hydroxyl groups on the aromatic ring (Stalikas, 2007) (see figure 2.6 (A)). Flavonoids in contrast, possess at least two phenol subunits and the compounds made up of three or more phenol subunits are called tannins. The basic flavonoid structure possesses a flavan nucleus that, consists of 15 carbon atoms arranged in three rings (C6-C3-C6), labelled as A, B and C in figure 2.6 (B) (Stalikas, 2007).

(A)



Name	R ₁	R ₂	R ₃	R ₄
Benzoic acid	H	H	H	H
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH ₃	OH	H
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH ₃	OH	OCH ₃
Gentisic acid	OH	H	H	OH
Veratric acid	H	OCH ₃	OCH ₃	H
Salicylic acid	OH	H	H	H



Name	R ₁	R ₂	R ₃	R ₄
Cinnamic acid	H	H	H	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	H	H
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	OCH ₃	OH	H
Sinapic acid	H	OCH ₃	OH	OCH ₃
Caffeic acid	H	OH	OH	H

(B)

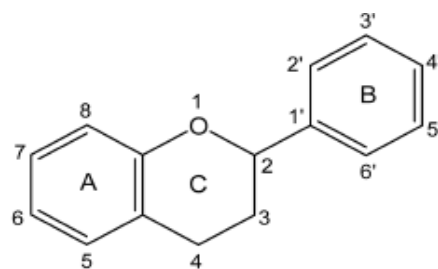


Figure 2.6. (A) Structures of commonly occurring phenolic acids and their substitution patterns and (B) Basic structure of flavonoids (Stalikas, 2007)

PPs are amongst the most heterogeneous and ubiquitous groups of natural compounds universally distributed among vascular plants and hence are an integral part of human diet (Alonso-Salces et al., 2001). As antioxidants, PPs function as free-radical scavengers, thereby limiting the incidences of numerous degenerative diseases associated with oxidative stress. PPs, in addition to imparting nutritional benefits, play a vital role in the organoleptic and commercial properties of plant-derived foods, since they contribute sensory attributes to the foods like colour, astringency, bitterness and overall flavour (Alonso-Salces et al., 2001).

The major PPs in apple include flavonols (quercetin glycosides), cinnamic acids (chlorogenic and caffeic acids), flavanols (catechin and epicatechin), procyanidins, dihydrochalcones (phloridzin) and anthocyanidins (cyanidin glycosides) (McGhie et al., 2005, Sun-Waterhouse et al., 2011b). Apples contain higher concentrations of unbound or free polyphenols relative to other commonly consumed fruits that remain available for interaction with other molecules (Sun et al., 2002). The total extractable PP content ranges from 110 to 357 mg/100g of fresh apples depending on variety (Liu et al., 2001, Podsedek et al., 2000). Figure 2.7 illustrates some of the commonly found PPs in apples.

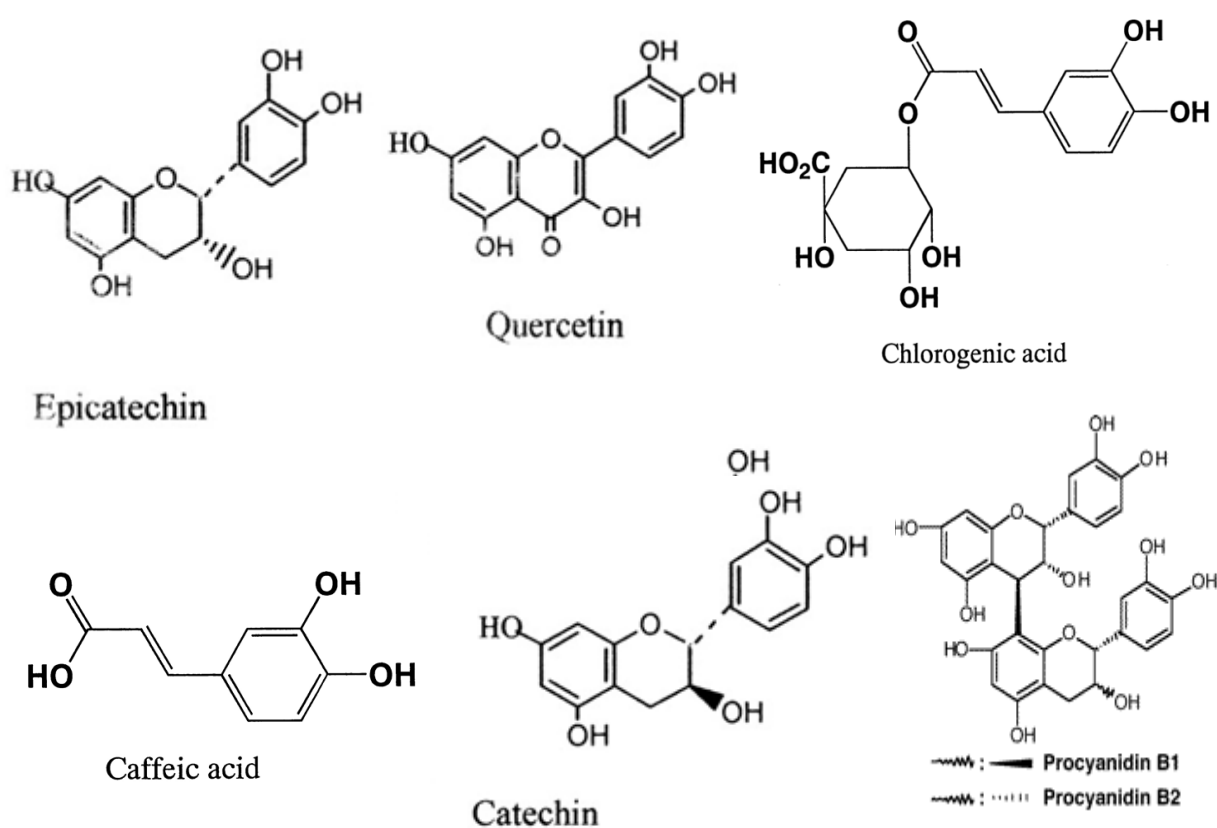


Figure 2.7. Some commonly occurring polyphenols in apples (Azuma et al., 2000, Boyer and Liu, 2004, Liu, 2004, Shoji et al., 2006).

The amount of PPs in apple is also reported to differ between its flesh and skin, with skin possessing a higher total polyphenolic content than the flesh (Boyer and Liu, 2004, Escarpa and Gonzalez, 1998, Sun-Waterhouse et al., 2011b, Wolfe et al., 2003). PPs commonly occurring in apple skin are procyanidins, catechin, epicatechin, chlorogenic acid, phloridzin and quercetin conjugates. Despite the presence of catechin, procyanidin,

epicatechin and phloridzin in apple flesh, these compounds tend to occur at a much lower concentration as compared with apple skin (Boyer and Liu, 2004). Quercetin conjugates have been reported to occur particularly in apple skin, whereas apple flesh tends to possess a higher concentration of chlorogenic acid than skin (Escarpa and Gonzalez, 1998).

Apple skin has been demonstrated to have a higher antioxidant activity and bioactivity than flesh, owing to the greater amount of antioxidant compounds, especially quercetin (Eberhardt et al., 2000, Wolfe et al., 2003). The studies determined a higher antioxidant activity of apples with peels in contrast with apples without peels. Vitamin C accounted for less than 0.4% of the total antioxidant activity, suggesting the major contributors of antioxidant activity were of PPs. Apples with intact skin have been found to markedly inhibit human liver cancer cell proliferation compared with peeled apples without its skin (Eberhardt et al., 2000). A further study by Wolfe et al. (2003) confirmed this observation by illustrating the pronounced efficacy of apple peels in inhibiting liver cancer cells as compared with other apple components.

Wolfe et al. (2003) reported that apple skin possess two to six times more PP content than its flesh, and two to three times more flavanoids in skin than in flesh. The antioxidant activity of apple skin was evaluated to be two to six times greater than its flesh in a study by Awad and de Jager (2000). These results substantiate the study by Leontowicz et al. (2003) who confirmed higher inhibition of lipid peroxidation and greater plasma antioxidant activity of apple skin in rats when compared with apple flesh. Apple skin is however, a waste product of apple sauce and canned apple manufacture. 16 million pounds of peels were estimated to be generated in this manner in New York state alone in 2000 according to the reports by the National Agriculture Statistics Service (Wolfe et al., 2003). Since, it is evident that apple peels may possess higher bioactivity than flesh, the potential of using bioactives in apple skin to improve health needs to be investigated.

2.3.3 Health benefits of apple consumption

Consumption of apple on a daily basis showed a significantly reduced risk of lung cancer in females in a study by Feskanich et al. (2000). In a similar study, intake of apples has been attributed to decreased events of lung cancer in both men and women (Marchand et al., 2000). The flavonoid content of apples was strongly correlated with the reduced incidence of lung cancer in Finnish population (Knekt et al., 1997). An investigation by Gallus et al. (2005) determined inverse association between apples and risk of ovarian, prostate and breast cancers.

The consumption of apples have been related with the drop of both cardiovascular disease and cardiovascular incidence and the effect was associated with the flavonoid content of apples (Hollman and Katan, 1999). Flavonoid quercetin has also been associated with reduced events of thrombotic stroke (Knekt et al., 2000). Hertog et al. (1993) studied the relationship between flavonoids and the risk of coronary heart disease in elderly population and found considerable correlation with a reduced mortality from heart conditions. In their study apple consumption contributed to about 10% of the total ingested flavonoids and attributed to the decline of deaths from coronary heart conditions.

Apple and pear intake by Australians have been reported to be responsible for subsidence of asthma and bronchial hypersensitivity (Woods et al., 2003). In the U. K., decline in asthma was attributed to apple consumption, while other fruits and vegetables were weakly correlated in a case-control study by Shaheen et al. (2001). Vitamins C and E were not associated with the protective effect in this study, suggesting the effect of apple flavonoid on the subjects screened. Catechin intake through consumption of apple and pear have also been linked to a positive effect on pulmonary function in Netherland adults (Tabak et al., 2001).

Apples have also been related to decreased events of Type II diabetes in the Finnish population and the effect has been linked to quercetin (Knekt et al., 2002). About 400 hypercholesteremic patients in Brazil showed significant weight loss after the consumption of apples three times a day over a 12 weeks period (Conceicao de Oliveira et al., 2003). In addition to epidemiological investigations, in vitro studies have also supported the positive action of apple polyphenols on various diseases.

The antioxidant activity of apple peels have been demonstrated to hinder the growth of liver and colon cancer (Eberhardt et al., 2000, Wolfe et al., 2003). Moreover, the dose dependent efficacy of apple extracts in preventing the proliferation of colon and liver cancer cells was demonstrated by Eberhardt et al. (2000). They suggested that natural antioxidants from fresh fruits could be more effective than a dietary supplement and the unique combination of polyphenols in apples accounted for the effect. Apple was noted to possess pronounced antiproliferative and antioxidant activity in comparison with 11 other commonly consumed fruits (Sun et al., 2002). The effectiveness of apples with skin to inhibit growth of HepG2 (liver hepatocellular carcinoma) liver cancer cells was found to be pronouncedly higher than apples without skin (Liu et al., 2001). Wolfe et al. (2003) confirmed the previous conclusion by demonstrating the significantly higher efficacy of apple skins alone to inhibit HepG2 cell proliferation than whole apples. The protective action of apples against several diseases has been attributed mainly to their antioxidant properties (Sun et al., 2002).

2.3.4 Apple-derived polyphenol bioactivity, bioavailability and metabolic pathway

The bioavailability and absorption of PPs in the body is of great importance in order to receive the potential health effects. The information on the absorption of PPs in the GI tract and their retention in the body from whole foods is scant (Bravo, 1998). The absorption of PPs in the intestine has been reported to be highly variable, often slow, and largely incomplete (Scalbert et al., 2002). The absorption and metabolism of dietary PPs depend exclusively on their chemical structure, which in turn is dependent on the degree of glycosylation/acylation, their basic structure (i.e. benzene or flavones derivatives), conjugation with other phenolics, molecular size, extent of polymerization and solubility (Bravo, 1998).

DuPont et al. (2002) investigated the bioavailability of PPs from apples and apple products in humans. No quercetin was detected in the plasma after the intake of 1.1 litres of apple cider. Very low amounts of 3'-methyl quercetin and 4'-methyl quercetin were found instead within one hour of apple cider consumption. Caffeic acid was noted to be absorbed at a fast rate into the plasma, but was undetectable within 90 min. Catechin, epicatehin and phloretin were not found in the plasma or the urine of the subjects in the study. Despite the

high antioxidant and antiproliferative activity of quercetin, its absorption in humans from whole foods is reported to be low by Peter Hollman et al. (1997). In their study, a quercetin bioavailability from apples was only 30% as measured from the plasma of the volunteers.

Walle et al. (2000) studied the bioavailability of quercetin and quercetin glycosides in regards to its absorption, and found that quercetin primarily existed as the aglycone form in the ileostomy fluid. β -glucosidases was thought to hydrolyse quercetin glycosides to quercetin, which then follows passive transportation and absorption. Further investigation in this regard by Day et al. (2003), affirmed that quercetin glycosides tend to be aglycosylated by lactase phlorizin hydrolase followed by its entry into the cell. Some intact glycosides however, are believed to be transported via sodium-dependent glucose transporter (SGLT1) pathway, wherein the glucosides were deglycosylated within the cell by cytosolic β -glucosidases. Quercetin-3-glucoside was reported to follow lactase phlorizin hydrolase (LPH) pathway and not the SGLT1 transport, while, quercetin-4-glucoside utilised both pathways. Apples contain some quercetin-3-glucoside, which, by following LPH pathway, would be available for intestinal uptake. However, apples also contain quercetin rhamnosides, xylosides and galactosides which are not readily hydrolysed by the LPH, and most likely are not easily absorbed by intestinal cells. Quercetin in onions, on the other hand, has been reported to occur in the form of quercetin glucosides and free quercetin, which are more available for intestinal uptake (Boyer and Liu, 2004).

Phloridzin, a glucoside conjugate of phloretin found in apples is also believed to be hydrolysed by the LPH pathway and the phloretin aglycone is absorbed by the intestinal cells (Crespy et al., 2001). Catechin and epicatechin have been reported to be absorbed by the small intestine epithelial cells. Unlike quercetin, epicatechin was found to be sulphated by human liver intestinal cytosols suggesting sulfation as the principal pathway for epicatechin metabolism (Vaidyanathan and Walle, 2002). The majority of flavanoid aglycones in general, pass through the intestinal epithelial cells where they are further conjugated. The flavanoid glycosides may be absorbed in small concentrations, however, the majority of the absorption takes place after their hydrolysis by intestinal hydrolases, such as LPH (Boyer and Liu, 2004).

Thus, considerable amounts of dietary PPs tends to remain unabsorbed in the gut lumen. The occurrence of a large number (10^{10} to 10^{11} cfu/g colonic content) of commensal and probiotic bacteria in the colon offers an opportunity for interaction between PPs and the

complex gut microbiota (Parkar et al., 2008). Gut microbial flora are known to act on and degrade PPs, ultimately mediating their rapid absorption by gut cells. *Enterococcus casseliflavus* and *Eubacterium ramulus*, microorganisms isolated from human faeces, have been found to degrade quercetin-3-glucoside as a source of carbon and energy (Schneider et al., 1999). In their study, *E. casseliflavus* appeared to utilise the sugar moiety of the glucoside, while, *E. ramulus* degraded the aromatic ring system to produce an intermediate, phloroglucinol. Similarly, bulk chlorogenic acid reaching the large intestine has been found to be metabolised by the gut microorganisms in rats (Gonthier et al., 2003). In humans, microbial metabolism converted about half of the ingested chlorogenic acid to hippuric acid (Olthof et al., 2003). The microbial transformation of PPs has been reported to generate metabolites with positive physiological significance (Parkar et al., 2008). For instance, soy isoflavone diadzein is microbially converted to equol, which imparts a more pronounced estrogenic action than the precursor itself (Bowey et al., 2003). Human colonic microflora has been shown to degrade dietary PPs (hydrocinnamates, flavanones and flavonols) *in vitro* following the metabolic events after cleavage of the ester or glycosidic bond (Rechner et al., 2004). The hydrolysis of glycosides has been reported to generate metabolites that possess higher bioactivity than the original parent compound. Bacterial transformation of aglycones in contrast generate more or less active compounds depending on the substrate being metabolized and the products formed (Rechner et al., 2004, Selma et al., 2009).

2.3.5 Application of polyphenols in functional foods

The health-benefiting antioxidant activity of PPs justifies their increasing usage in functional foods application. Efforts are made to incorporate PPs in various food systems as a convenient way to deliver health benefits of PPs. Functional fruit juices or drinks containing PPs and other bioactive components are gaining popularity due to added health benefits (Sun-Waterhouse, 2011). Pomegranate juice and red wine marketed as polyphenol-rich beverages in US has been shown to have strong antioxidant activity and related health benefits (Seeram et al., 2008). The clinical study by Heber et al. (2007) demonstrated significant reduction of thiobarbituric acid reactive substances (TBARS) linked with cardiovascular diseases in human with the consumption of pomegranate juice. Large number of other fruits including cranberry, blueberry, apple, blackcurrant, acai, grapes, kiwifruits, and cherries are also employed to produce polyphenol-rich beverages (Seeram et al., 2008, Sun-Waterhouse, 2011).

Commercial juice concentrates of chokeberry, blackcurrant and elderberry was characterized for their antioxidant activity and were suggested to be used as potential functional ingredients for juices (Bermudez-Soto and Tomas-Barberan, 2004). The new trend of incorporating PPs in the diet involves the usage of minimally processed fruit preparations such as juice and pomace that are rich in PPs. Such PP rich fruit products and wastes find their application in smoothies, yoghurts, ice-creams and breads.

PPs have been known to impart bitterness and astringency to the product (Axten et al., 2008, Lesschaeve and Noble, 2005) which tends to impose a huge challenge for functional product development with acceptable sensory attributes. Moreover, the enriched functional foods need to retain these bioactives at a required benefiting levels in the finished products (Gray et al., 2003, Sun-Waterhouse et al., 2010a). Polyphenol and fibre rich smoothies developed by Sun-Waterhouse et al. (2010a) received lower consumer acceptability. Flavour, texture and overall acceptability were reported to decrease with increase in content of fibre and PPs. Corn breakfast cereals enhanced with fruit powders (Blueberry, cranberry, grape and raspberry) extruded on the laboratory scale were determined to contain low antioxidant activity (Camire et al., 2007). The utilisation of low amounts of fruit powders was associated with low antioxidant activity in their study.

A sound knowledge about the effects of added bioactives including PPs on the properties of the final product and the ways to overcome these effects is important for successful formulation (Sun-Waterhouse, 2011). Sun-Waterhouse et al. (2011a) successfully developed an ice cream using aqueous fractions from purees of kiwifruits with no commercial flavouring and coloring agents. The amounts of PPs and the related antioxidant capacity of the final ice cream were reported to be high with good rheological properties. Apple PPs added to drinking yoghurts have also been evaluated for their influence on the growth of yoghurt culture (Sun-Waterhouse et al., 2011e). They suggested incorporation of PPs to yoghurts before fermentation in order to achieve higher total polyphenolic content in the final product as well as to increase the growth of yoghurt bacteria. The apple polyphenol enriched model milk drinks developed by Wegrzyn et al. (2008) demonstrated good physical stability with a substantial antioxidant activity.

PPs are being investigated for their application in baked products particularly bread. Bread making is a complex process and the processing methods can modify the polyphenol

content in the final bread in several ways (Manach et al., 2004). Consequently, it presents a technological challenge in terms of retaining the functionality of the added PPs in the finished products. Sun-Waterhouse et al. (2009a) successfully developed a gluten-free bread using aqueous extract of green-fleshed kiwifruit with high phenolic content, and good stability. The bread was well accepted by the sensory panel and had smoother and softer texture compared with the plain gluten-free bread. In another study, a significant drop in polyphenol concentration and antioxidant activity (85-90%) was reported in buckwheat bread after baking compared with bread made with wheat flour (Vogrincic et al., 2010). The levels of rutin were determined to decrease during the bread making process while the stability in quercetin levels was observed in their study. The addition of water and yeast to the flour resulted in the transformation of 85% of rutin into quercetin. These observations substantiates with that of Dietrych-Szostak and Oleszek (1999), who concluded that thermal processing of buckwheat renders detrimental effect on its flavonoid content. The employment of different PP source, quantity and processing methods might be the reasons for the discrepancies in these studies. Application of PPs in extended-shelf life products has also been investigated. Snack bars prepared using apple polyphenol extract was evaluated to contain considerable levels of phenolic in the finished products (2.87 and 2.22 mg catechin equivalent/g of bar) compared with the control bar (1.45mg catechin equivalent/g bar) after baking (Sun-Waterhouse et al., 2010b). Successful functional product development hence, requires information on the interaction of ingredients coupled with the effect of processing methods on the added components.

The demand and popularity of natural antioxidants to increase oxidative stability especially in lipid-rich foods has provided a new dimension to polyphenol application (Frankel, 1993). For instance, green tea and grape seed extracts rich in PPs have been demonstrated to increase the shelf life of low sulphite raw beef patties by delaying lipid oxidation and microbial spoilage (Banon et al., 2007). These polyphenolic rich extracts also delayed the onset of rancid flavour in cooked patties in their study. The antioxidant capacity of PPs or PP-rich fruit extracts has been affirmed to extend the shelf life of the products stored under retail display conditions including fish oil and frozen fish (Pazos et al., 2005), cooked pork patties (Nissen et al., 2004) and cooked turkey (Mielnik et al., 2006). The ability of polyphenol antioxidants to lower, delay or inhibit lipid oxidation was linked to the increase in shelf life of these products. Polyphenols including quercetin (200 ppm), myricetin (200 ppm), tannic acid (30 and 200ppm) and ellagic acid (30 and 200ppm) have been shown to inhibit

lipid oxidation in raw fish (Ramanathan and Das, 1992). Investigation by Sun-Waterhouse et al. (2011d) demonstrated the efficacy of caffeic acid to increase the stability of olive oil using microencapsulation method. The final oil product was not only reported to have good oxidation stability but nutritional value with increased levels of polyphenolic content and unsaturated fatty acids. Thus, the use of PPs present dual benefits of inhibiting lipid oxidation as free radical scavenger while also enhancing the nutritional value of the final product (Sun-Waterhouse et al., 2011d).

2.4 Studies on the food systems containing both polyphenols and probiotic bacteria

The physiological effects of PPs and PB individually on humans are well documented and established, but, studies involving the interaction of PPs and PB, as well as their synergistic effects on humans, are scarce. There is some evidence that PPs may have some influence on the colonic bacteria as well as PB.

The influence of common dietary PPs on the growth of human gut bacteria and their adhesion to enterocytes was investigated by Parkar et al. (2008) with a view to gain better understanding of their interaction. The probiotic *L. rhamnosus* was less affected by the PPs tested in their study while, the PPs were most active in inhibiting enteropathogen *S. aureus* and the commensal *E. coli*. The workers further concluded that in general, flavonols, isoflavones and glycosides tend to display low antibacterial activity against *Lactobacilli*. Moreover, naringenin and phloridzin reduced the adherence of *S. typhimurium* to Caco-2 enterocytes. In contrast, phlorodzin and rutin improved the adhesion of probiotic *L. rhamnosus*. Thus, PPs have been reported to possess the capability to modify micro-flora in the gut, which in turn may impart positive health effects on the gut (Parkar et al., 2008). Fruit extracts of blueberry, feijoa, and strawberry were also demonstrated by Sophorn (2010) to stimulate growth of probiotic species including *Bifidobacterium* and *Lactobacillus* in dose-dependent manner. Moreover, fruit extracts in their study exerted inhibitory effects on pathogenic *Listeria monocytogenes*. In another study by Molan et al. (2009b), the addition of water extracts of two blueberry cultivars to broth media containing pure cultures of *L. rhamnosus* and *B. breve* resulted in a pronounced increase in the population of these strains. Furthermore, oral administration of these extracts (4 mL/kg/day) for 6 days to rats resulted in a marked positive influence on the population size of lactobacilli and bifidobacteria in their

study. They further concluded that blueberry extracts has the potential to improve gut health by increasing the numbers of beneficial bacteria. The aqueous extracts of apple, banana and orange has been determined to improve the growth of probiotic *Lactobacillus* and *Bifidobacterium* strains and inhibit pathogenic bacteria (Sutherland et al., 2009).

The commercial apple polyphenol extract has also been demonstrated to facilitate the growth of *Streptococcus* and *Lactobacillus* in the drinking yoghurts (Sun-Waterhouse et al., 2011e). Purified PPs in their study have been reported to impart different effects on the yoghurt bacteria. Chlorogenic acid increased the growth of *Streptococcus* but reduced the *Lactobacillus* colonies considerably in the yoghurts. Phlorizin in their study was observed to cause a marked reduction in the *Lactobacillus* colonies and a minimal change in the growth of *Streptococcus* was noted. They further concluded that PPs exert varied effects (stimulatory, minimal or inhibitory effects) on the probiotic growth depending on its concentration, structure and probiotic strain used. Duda-Chodak et al. (2008) observed stimulation in the growth of *L. casei* with catechin at a concentration of 100-400 μ M and chlorogenic acid at a concentration of 400 μ M. Contrastingly, quercetin at a concentration 25-50 μ M showed inhibitory effect on the growth of probiotic bacteria.

Selenium-containing green tea extracts (10%-25%) with high polyphenolic content has been shown to significantly increase the numbers of *L. rhamnosus* and *B. breve* recovered from the batch fermentation (Molan et al., 2009a). Lee et al. (2006) investigated the effect of phenolic components of tea extract on different intestinal bacteria. This study determined that the different strains of intestinal bacteria exhibit varying degree of growth sensitivity to tea phenolics. The growth of certain pathogenic bacteria including *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides* spp. was markedly inhibited. In contrast, the growth of commensal *Clostridium* spp. as well as probiotic *Bifidobacterium* spp. and probiotic *Lactobacillus* spp. were less severely affected by the tea phenolic extracts. The faecal homogenates containing bacteria in their study were also demonstrated to catalyse tea phenolics including epicatechin, catechin, 3-O-methyl gallic acid, gallic acid and caffeic acid to produce aromatic metabolites depending on the bacterial species. They further hypothesised that tea phenolics influence the intestinal environment by modulating intestinal micro-flora, probably by acting as metabolic prebiotics. This indicates that the bioactivity of different PPs may play an important role in maintenance of gastrointestinal health.

PPs have been recently established to bind to the bacterial surface including *Lactobacillus*, *Bifidobacterium* and *Streptococcus* species, and significantly enhance their total oxidant-scavenging capacities (Koren et al., 2009). The increased antioxidant capacities of PB might improve the protection of human cells against oxidative damage generated during infections and inflammation. Two polyphenolic compounds, gallic acid and catechin have been analysed to promote the survival of probiotic *Streptococcus thermophilus* at pH 3.0 and 4.0 (Khalil, 2010). The catechin-adapted probiotic cells exhibited pronounced tolerance to oxgall, and the bacteriocins produced by PP-adapted probiotic cells markedly inhibited the proliferation of *E. coli* O157:H7, *P. aeruginosa* and *L. monocytogenes*.

Thus, there exist possibilities to establish a combination of polyphenols and probiotic bacteria for utilising the PPs to selectively stimulate the proliferation and/or activity of probiotics. Moreover, the synergistic combination of polyphenols and probiotics may provide an opportunity to produce cells with enhanced attachment ability and tolerance to environmental stresses.

Chapter 3

Preparation and characterisation of physicochemical properties of apple skin extracts and their derived milk drinks

3.1 Introduction

As addressed earlier in chapter 2 (section 2.3.2), apples have been determined to contain substantial amounts of polyphenols (PPs) including flavonols, cinnamic acids, dihydrochalcones and anthocyanidins (McGhie et al., 2005) that play a vital role in prevention of various chronic diseases (Boyer and Liu, 2004, Sun-Waterhouse et al., 2011b). About 46% of the polyphenolics in whole apple were reported to be from the skin by McGhie et al. (2005). Apple skin contains relatively higher amounts of PPs (Sun-Waterhouse et al., 2011b, Wolfe et al., 2003), which contribute to the detected antioxidant and antiproliferative activities, to a much greater extent than those in apple flesh (Eberhardt et al., 2000). In our study, apple skin was used as a source of polyphenols. Apple was selected for its regular consumption among different cultures and its evident nutritional benefits.

Many sample preparation methods have been developed to generate PP extracts, typically including milling, grinding and homogenisation, drying and extraction steps. Drying methods include air-drying or freeze-drying. Extraction is a vital step for recovering and isolating target bioactives from plant material, prior to analysis. Extraction efficiency and extract composition can be greatly influenced by the physicochemical characteristics of the material for extraction (e.g. particle size), matrix components (e.g. co-existing PPs or non-PPs including interfering substances), extraction method (e.g. aqueous or ethanolic extraction, and extraction temperature and pressure applied) and chemical structure of target PPs (Alonso-Salces et al., 2001, Stalikas, 2007).

PPs range from simple phenolic monomers to complex polymeric tannins. PPs carry multiple hydroxyl groups that can be conjugated to sugars, acids or alkyl groups that

influences their polarity (Luthria, 2008). The pH of the extraction solution governs the extent of solubility for soluble compounds and also influences the possible solubilisation of the hydrolysable fraction (Escribano-Bailon and Santos-Buelga, 2003). The extraction solvents typically used for extracting of PPs present in apples include alcohols (e.g. methanol and ethanol), acetone and ethyl acetate (Guyot et al., 1997, Lister et al., 1994). The inefficiency of pure organic solvents in extracting very polar PPs leads to the requirement for the addition of water (Stalikas, 2007). Moreover, solvents like acetone and ethyl acetate have been reported to generate low yields of PPs (Escribano-Bailon and Santos-Buelga, 2003, Fernandez de Simon et al., 1990). Methanol, these solvents were not suitable for producing food grade extracts for human consumption, owing to the potential toxicity of the residual solvents (Nawaz et al., 2006). Therefore, acidified water and aqueous ethanolic solutions were used in this current study. The pH of 3 was set for the acidified water in order to maintain the extracted PPs in a nearly native state under which the apple PPs and organic acids co-exist. It was reported that the pH of the apple juice is approximately 3 (Picinelli et al., 1997).

Accelerated solvent extraction (ASE) technology uses organic solvents at high pressures and temperatures above the boiling point (Richter et al., 1996), which allows reduced volume of extraction solvent, shorter analysis time and less handling to produce accurate results (Alonso-Salces et al., 2001). Good recovery and repeatability for the extraction of PPs from apple peel, pulp and derived food products using ASE has been reported (Alonso-Salces et al., 2001, Sun-Waterhouse et al., 2011b, Sun-Waterhouse et al., 2010b).

This chapter describes the evaluation of the polyphenolic composition of apple skin extracts prepared using two different preparation methods: ethanolic and aqueous methods. Total extractable polyphenol content (TEPC) and individual polyphenols were analysed using Folin-Ciocalteu assay and HPLC respectively. The antioxidant activities of the two extracts were determined by FRAP (Ferric reducing antioxidant power) assay. Vitamin C and uronic acid content of the two apple skin PP extracts was also determined. The TEPC content of the milk drinks enhanced with the PP extracts was determined following the extraction using ASE.

3.2 Materials and Methods

3.2.1 Chemicals and materials

The chemicals and materials used are listed in Table 3.1.

Table 3.1 Chemicals and materials used in this study

Chemicals and Materials	Manufacturer
White fleshed apples	Plant & Food Research, Hawke`s Bay, New Zealand
Food-grade ethanol (96%)	Anchor Ethanol Ltd, Tirau, New Zealand
Sodium hydroxide, absolute ethanol	Merck, Darmstadt, Germany
Celite®	Maville Service Corporation, USA
Food-grade citric acid	Hawkins Watts Ltd., Auckland, New Zealand
Sulfamic acid, sulphuric acid, methanol, <i>n</i> -hexane, acetone	Ajax Chemical Ltd, Sydney
Instant skim milk powder	Pam`s, New Zealand. Batch no: B2816062, purchased from New World supermarket, Auckland
Catechin, epicatechin, phloridzin, p-coumaric acid, phloretin, quercetin, ferulic acid, naringin, D-galacturonic acid, <i>m</i> -hydroxydiphenyl, Folin-Ciocalteu phenol reagent, sodium acetate, glacial acetic acid, tripyridyltriazine, trolox, sodium hydrogen carbonate, sodium hexametaphosphate, dichlorophenol indophenol, ascorbic acid, sodium hydroxide	Sigma-Aldrich, St Louis, MO, USA

3.2.2 Preparation of apple skin polyphenol extracts

White fleshed apples from Plant & Food Research's breeding programme were harvested between March and April 2010 in Hawke's Bay, New Zealand, and delivered to Plant & Food Research in Auckland, New Zealand the morning following picking. The apples were stored at 2 °C prior to skin extract preparation. The apples were peeled and the skin was stored in a freezer (-80 ± 3 °C, Thermo Electron Corp., Revco, Cambridge, UK) and freeze dried (Telstar Cryodos-80 Freeze Drier, Telstar Industrial, SL, Terrassa, Spain). The freeze-dried skin was then ground to fine powder using a ring grinder (Bench Top Ring Mill, Rocklabs Ltd, Auckland, New Zealand) and subjected to extraction using either 96% v/v food grade ethanol or citric acid infused water as follow:

3.2.2.1 Ethanolic extraction

The extraction of PPs from the apple skin was performed using Accelerated Solvent Extractor (ASE300, Dionex Corp., Sunnyvale, CA, USA) following the method of Sun-Waterhouse et al. (2011b). The ground skin sample (4 g) was mixed homogeneously with 4 g Celite® (diatomaceous earth). The resultant mixture was then packed into Dionex standard 34 mL stainless steel extraction cells and subjected to extraction with 96% v/v food-grade ethanol at 40 °C under a constant stream of nitrogen gas at 1500 psi, with 5 min of heating and 10 min of static time. The PP extract obtained was then concentrated with Rapidvap® (Labconco, Kansas, USA) at 40 °C for 70 min and was subjected to further concentration in Labconco CentriVap® concentrator (Model 78100–01, Ultra-Low Cold Trap, Kansas, USA) at 40 °C for 3 h. The concentrated PP extract was then freeze-dried (Telstar Cryodos-80 Freeze Drier, Telstar Industrial, SL, Terrassa, Spain) (appendix III) and stored at -80 °C until required for further analyses.

3.2.2.2 Aqueous extraction

Aqueous extraction of PPs from apple skin was performed following the method of Sun-Waterhouse et al. (2011b) with modifications. Acidified water was prepared by dissolving approximately 1 g food grade citric acid in 100 mL Milli-Q^{PLUS} water (Millipore Water Purification System, Merck Millipore, USA) until the solution reached pH 3. This acidified water solution was then used as an extraction medium. An aliquot (1 g) of freeze-dried apple skin powder was mixed with 10 mL of acidic water (pH 3) and the mixture was then subjected to vortexing followed by centrifugation at 4400 rpm for 3 min twice. The clear supernatant was then freeze dried (Telstar Cryodos-80 Freeze Drier, Telstar Industrial, SL, Terrassa, Spain) (appendix III) and stored at -80 °C until required.

3.2.3 Analyses of apple skin polyphenol extracts

3.2.3.1 Total extractable polyphenol content analysis of apple skin extracts

A Solid Phase Extraction (SPE) column was used to pre-treat both ethanolic and aqueous skin extracts prior to the total extractable polyphenolic content (TEPC) analysis to eliminate the potential interference of sugar and ascorbic acid with the Folin-Ciocalteu assay as recommended by Sun-Waterhouse et al. (2011b). Before pre-treatment, ethanolic and aqueous skin extracts (30 mg each) were reconstituted in 25% methanol (3 mL) to give a concentration of 10 mg/mL. The first eluate from SPE was used for vitamin C analysis (see 3.2.3.4). The subsequent eluates resulting from each flush with 95% methanol (in an aliquot of 3 mL) were collected for TEPC analyses and antioxidant analyses (see 3.2.3.3).

The Folin-Ciocalteu (FC) reagent was used to determine the TEPC of the apple skin extracts following the method of Singleton et al. (1999). An aliquot (1 mL) of 0.2 M FC reagent was added to 200 μ L of eluate of either the ethanolic or aqueous skin extracts after SPE treatment, in 20 mL Falcon screw cap test tubes. An aliquot (800 μ L) of 7.5% (w/v) sodium carbonate solution was then introduced into each tube no earlier than 3 and no later than 8 min after addition of FC reagent. The tubes were capped and incubated in the dark for 1.5 h before recording the absorbance at 760 nm with a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, USA).

A standard curve was prepared (as shown in appendix V) by preparing a standard solution of 0.5 mg/mL catechin in methanol (25% v/v) and diluting to final concentrations of 0.0000, 0.0250, 0.03750, 0.0500, 0.0625, 0.0750, 0.1000 and 0.1250 mg catechin/mL. Sample extracts were diluted to fit appropriately within the standard curve. The final results are expressed as mg catechin equivalent per g of dry extract (mg catechin eq./g extract).

3.2.3.2 Analysis of individual Polyphenols from apple skin extracts by HPLC

Individual PPs of the ethanolic and aqueous extracts (1 mL per test sample) were analysed in duplicate by HPLC (high-performance liquid chromatography) based on the procedure of Stevenson et al. (2006). Prior to analysis, the freeze-dried skin extracts (20 mg) were dissolved in 1 mL of 25% methanol and the mixture was centrifuged at 13,000 *g* for 5 min. A clear supernatant was achieved through filtration using a sterile Ministart® filter (0.45 µm, Sartorius Stedim Biotec, 13781 Aubagne Cedex, France) prior to filling into HPLC vials. The testing samples were analysed (at 280 and 350 nm wavelengths) using a Shimadzu analytical HPLC, equipped with a column oven (C40-10ASVP), auto-sampler (SIL-10AF), vacuum solvent degas module and diode-array detector (SPD-M10AVP), fitted with Synergi® Polar-RP ether-linked column (250×4.6 mm, 4 µm particle size, 80-Å ether-linked column, Phenomenex, Auckland, New Zealand). The mobile phases (A) acetonitrile + 0.1% formic acid and (B) acetonitrile/water/formic acid (5:92:3 by volume) were pumped at 1.5 mL/min at 45 °C. Injection volume was 40 µL. Individual PPs were identified based on their retention time and absorbance maximum (λ_{max}). An external standard of apple berry mix (50 ppm) was used to identify PPs. The standard apple berry mix contained catechin, caffeic acid, chlorogenic acid, *p*-coumaric acid, rutin, phloridzin, quercetin and phloretin.

The individual PPs in the two extracts were quantified by using naringin (50 ppm) as internal standard. The stock solutions of the extracts were prepared by dissolving 60 mg extracts in 1.5 mL of 25% methanol. After centrifugation at 13,000 *g* for 5 min, the supernatants were collected in separate Eppendorf tubes and 1 mL of the stocks were mixed with 1 mL of 100 ppm stock solution of naringin to give a final concentration of 20 mg/mL of extracts and 50 ppm of naringin.

3.2.3.3 Analysis of total antioxidant activity of apple skin extracts

The total antioxidant activity (TAA) of either the ethanolic or aqueous extract was estimated according to the procedure described by Benzie and Strain (1999). An aliquot (198 µL) of FRAP reagent was mixed with 10 µL of eluate of either the ethanolic or aqueous skin extracts after SPE treatment as well as with 25% methanol as the reagent blank in microtitre

plate (96-well flat bottom microplate, Greiner bio-one, Frickenhausen, Germany). The FRAP reagent was prepared freshly and warmed at 37 °C for 2 h before use. The FRAP reagent contained: 1) 10 mL, 300 mM acetate buffer (pH 3.6), 2) 1 mL, 10 mM tripyridyltriazine (TPTZ) and 3) 1 mL, 20 mM Ferric chloride (FeCl₃). The acetate buffer was prepared by mixing 1.9 g sodium acetate anhydrous to 16 mL glacial acetic acid and the final volume was adjusted to 1 L with DW. TPTZ solution was prepared by dissolving 0.156 g TPTZ in 50 mL DW and 172 µL concentrated hydrochloric acid. The absorbance of sample-reagent mixture was recorded at 593 nm wavelength after 20 min using a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, USA).

A standard curve was prepared (as shown in appendix VI) by making a standard solution of 0.25 mg/mL Trolox in methanol and diluting to final concentrations of 0.000, 0.025, 0.050, 0.075, 0.100, 0.150, 0.200 and 0.250 mg Trolox/mL. Sample extracts were appropriately diluted to fit within the standard curve. The final results are expressed as mg Trolox equivalent per g of dry extract (mg Trolox eq./g extract).

3.2.3.4 Determination of vitamin C of apple skin extracts

Ascorbic acid (vitamin C) in the two polyphenol extracts was determined using the AOAC titration method (AOAC, 1990). The first eluate resulting after SPE treatment of ethanolic and aqueous skin extracts were used for this analysis. Sodium hexametaphosphate stock (20 g/100mL), glacial acetic acid and DI water were mixed in a volume ration of 2:1:7 (v/v/v). 6 mL of the resulting solution was mixed with 1mL either ethanolic or aqueous skin extract eluate solution and titrated against titration solution. The titration solution contained 21 mg sodium hydrogen carbonate, 25 mg dichlorophenol indophenol in 100 mL DI water. Calibration was performed through titration, using as an ascorbic acid standard (1 mg/mL).

3.2.3.5 Determination of uronic acid content of apple skin extracts

The uronic acid (UA) contents of the ethanolic and aqueous skin extracts were determined (in duplicate) following the procedure of Sun-Waterhouse et al. (2010b) derived from the basic method of Filisetti-Cozzi and Carpita (1991). The apple skin extracts were

hydrolysed by mixing freeze-dried extracts (5 mg) with 1 mL sulphuric acid (H_2SO_4) in duplicate Kimax tubes. The tubes containing the sample acid solution were vortexed and allowed to stand for 5 min in an ice bath. 1 mL H_2SO_4 served as reagent control. Following five min incubation, 1 mL of H_2SO_4 was added to the tubes which were then vortexed and allowed to stand for a further 5 min in the ice bath. To this, 0.5 mL Milli-Q^{PLUS} was added twice and vortexed and allowed to stand for 5 min. The contents of the tubes were then diluted with 7 mL of water and vortexed. The sample tubes were then centrifuged at 3300 rpm for 10 min after transferring the contents to 15 mL centrifuge tubes.

For each extract hydrolysate, following centrifugation, triplicate samples of supernatant (400 μL each) from the sample tubes were transferred into separate 15 mL tubes where one of the three tubes served as the sample control. For reagent control, 400 μL supernatant was transferred in two 15 mL tubes. To all the supernatants 40 μL of 4M sulfamic acid/ potassium sulfamate solution (pH 1.6) was added. After vortexing, 2.4 mL of sodium tetraborate solution was added to the sulphuric acid solution followed by further vigorous vortexing. The tubes were then incubated in a boiling water bath (100 °C) for 20 min and then cooled by placing them into an ice bath for 10 min. To reagent control tubes and to two tubes for each sample hydrolysate, 80 μL of *m*-hydroxydiphenyl solution was added. For the sample control tube, 80 μL of 0.5% sodium hydroxide (NaOH) was added. All the tubes were vortexed to ensure adequate mixing of the contents. The absorbance was read after 30 min at 525 nm against the reagent control. The values of the sample controls were subtracted from the corresponding sample absorbances.

A calibration curve for D-galacturonic acid standard was established (as shown in appendix VII) by preparing a standard solution of 200 $\mu\text{g/mL}$ in MilliQ water and diluting to final concentration of 5, 10, 15, 20, 30, and 40 $\mu\text{g/mL}$ with MilliQ water. Sample extracts were appropriately diluted to fit within the standard curve. The final results were expressed as galacturonic acid percentage (GalA, %w/w).

3.2.4 Preparation and total polyphenolic content analyses of polyphenol-enhanced milk drinks

3.2.4.1 Preparation of polyphenol-enhanced milk drinks

Skim milk powder was first mixed with filtered RO (reverse osmosis) water to prepare skim milk with final concentration of 12% (w/v). The skim milk was then homogenised using a Silverson mixer (X screen, rpm Silverson Machines Inc., East Longmeadow, MA, USA) at 2000 rpm for 3 min. Polyphenol-Milks were prepared in duplicate by adding either ethanolic or aqueous PP extracts (1 g each) to skim milk (99 g) to give a final concentration of 1% (w/w). The mixtures were then gently mixed using a Silverson mixer at 1000 rpm for 2 min, followed by heat treatment at 85 °C for 30 min using the double boiler technique. The Schott bottles containing the milk drinks were wrapped with aluminium foil to avoid interference from the light with PPs.

3.2.4.2 Total extractable polyphenol content of apple skin polyphenol extract-enhanced milk drinks

The total extractable polyphenol content (TEPC) of apple skin PP extract-enhanced milk drinks was estimated. The preparation of PP extract from PP fortified milk was performed following the method of Sun-Waterhouse et al. (2011e) using ASE. The milk samples (5g) (with or without apple skin PP extracts) were mixed with Celite® (8.5 g) and packed into Dionex standard 34 mL stainless steel extraction cells. The samples were then subjected to three cycles of extraction using *n*-hexane (100%), acetone (100%) and then methanol (100%) sequentially under N₂ in a pressurised Accelerated Solvent Extractor operating at 40 °C and 1,500 psi, with 5 min heating and 10 min static time. The solvents carrying the extracted PPs were then concentrated using the Ultra-Low Cold Trap Centrivap® (Model 78100-01, Labconco Corp., Kansas City, MO), and dried using a freeze drier (Telstar Cryodos-80, Telstar Industrial SL, Spain). The prepared polyphenol extracts were kept at -80 °C. Prior to Folin-Ciocalteu assay for determination of TEPC, the extracts were reconstituted in 25% methanol (20 mg/2mL) and pre-treated using SPE. The first eluate

from SPE was discarded and the subsequent eluates resulting from each flush were collected for TEPC analysis.

3.2.5 Statistical analysis

Data were statistically analysed using Minitab® (version 16) software (Minitab Inc., State College, Pennsylvania, USA) with Two-sample *t*-test. At least two observations per analysis were performed and the results were expressed as mean values and standard deviation of the mean. The difference is considered to be of significant importance at more than 95% confidence interval ($P < 0.05$). The Ryan-Joiner test on residuals was used for the normality test (if $P > 0.1$, accept H_0 , data are normal) and Levene's test for the equal variances (if $P > 0.05$, accept H_0 , the variances are equal).

3.3 Results and Discussion

3.3.1 Polyphenol content determination and HPLC profiling of apple skin extracts

The PP extract preparation methods from apple skin significantly affected the detected TEPC as shown in figure 3.1. The Folin-Ciocalteu method indicated that the PP extract prepared using the aqueous method had significantly ($P < 0.05$) higher TEPC (21.44 ± 0.12 mg catechin eq./g dried extract) compared with ethanolic PP extract (20.66 ± 0.04 mg catechin eq./g dried extract). This result indicates that more total polyphenol content per gram of dried skin extract was generated using the aqueous method, compared with the ethanolic method. The results substantiates that of Sun-Waterhouse et al. (2011b). In their study, the apple fibres prepared using the aqueous method showed higher TEPC content than that prepared using ethanolic extraction. The apparent greater amount of TEPC in aqueous apple skin extract may be attributed to more cell wall-bound PPs being retained using aqueous method and to the engagement of PPs in cell wall cross-linking (Sun-Waterhouse et al., 2008a). The polar nature of phenolic as well as polyphenolic compounds allows them to be only moderately soluble in polar solvents such as ethanol and methanol. Moreover, close affiliation of polyphenols with hemicellulose polysaccharide in parenchyma cell walls (O'Neill and Selvendran, 1985) contribute to cell wall cross-linking, appearing as glycosides attached to one or more sugar moieties. These glycosides are more water soluble than their aglycone forms (Strack, 1997, Williamson et al., 2000) and hence, organic solvents as well as water are more suitable for their extraction (Sun-Waterhouse et al., 2008a). 21.44 mg polyphenolic phytochemicals per gram of dried skin extract were detected (Figure 3.1) by Folin-Ciocalteu assay from the extract obtained by the aqueous extraction method. In comparison, 20.66 mg polyphenolic phytochemicals per gram of dried skin extract were detected from the ethanolic extract. This is consistent with the other observations of the other workers, i.e. there are higher levels of polyphenols present in skin extract prepared using the aqueous method (Sun-Waterhouse et al., 2011b).

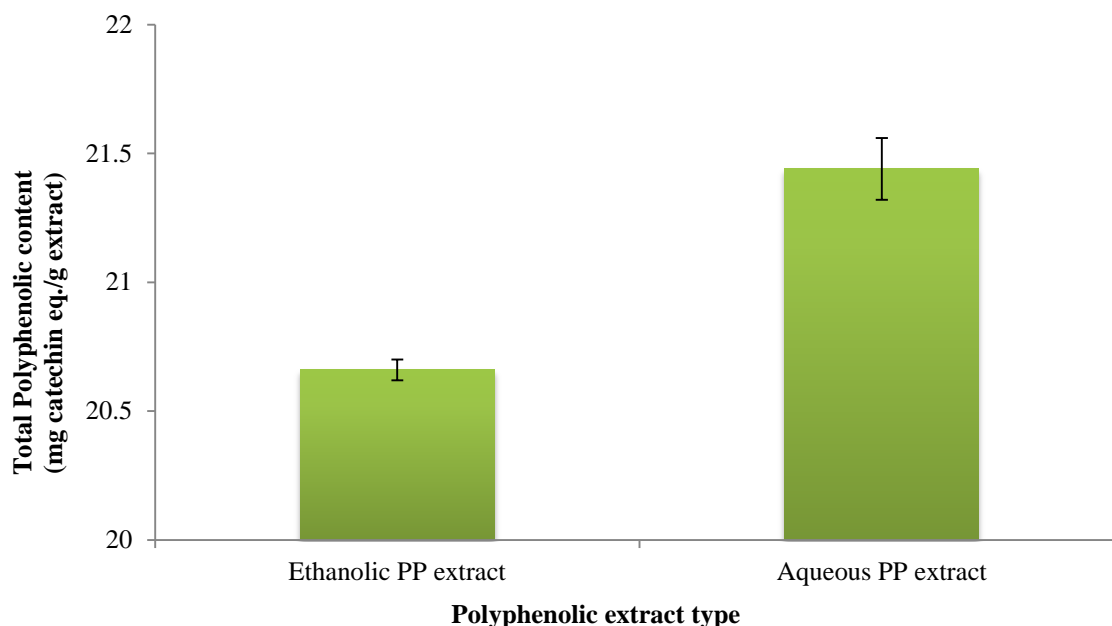


Figure 3.1. Determination of total extractable polyphenol content of ethanolic and aqueous polyphenol extracts

The apple skin extracts in the current study contained substantial amounts of total extractable polyphenol content, ranging from 20.66 - 21.44 mg catechin eq./g extract (Figure 3.1). This is comparable with the total extractable phenolic content of 6.9 - 11.5 mg/g of fresh skin in a study by McCann et al. (2007). The peels of four apple varieties were shown to contain higher polyphenol levels than the corresponding flesh in a study by Wolfe et al. (2003). In their study, the highest flavonoid content detected was in the apple peels of Rome Beauty variety (306.1 ± 6.7 mg catechin eq./100g peels). The difference in the amount of polyphenols in the extracts prepared by the two different methods is also reflected in the HPLC profile (figure 3.2) with higher quantity in the aqueous PP extract compared with the ethanolic PP extract. The PPs detected in the two extracts in the current study included catechin, caffeic acid, chlorogenic acid, epicatechin, *p*-coumaric acid, procyanindin trimer, *m*-coumaric acid, ferulic acid, *o*-coumaric acid, rutin, phloridzin, quercetin and quercetin derivative suggesting the presence of phenolic compounds commonly occurring in apples (Lee et al., 2003).

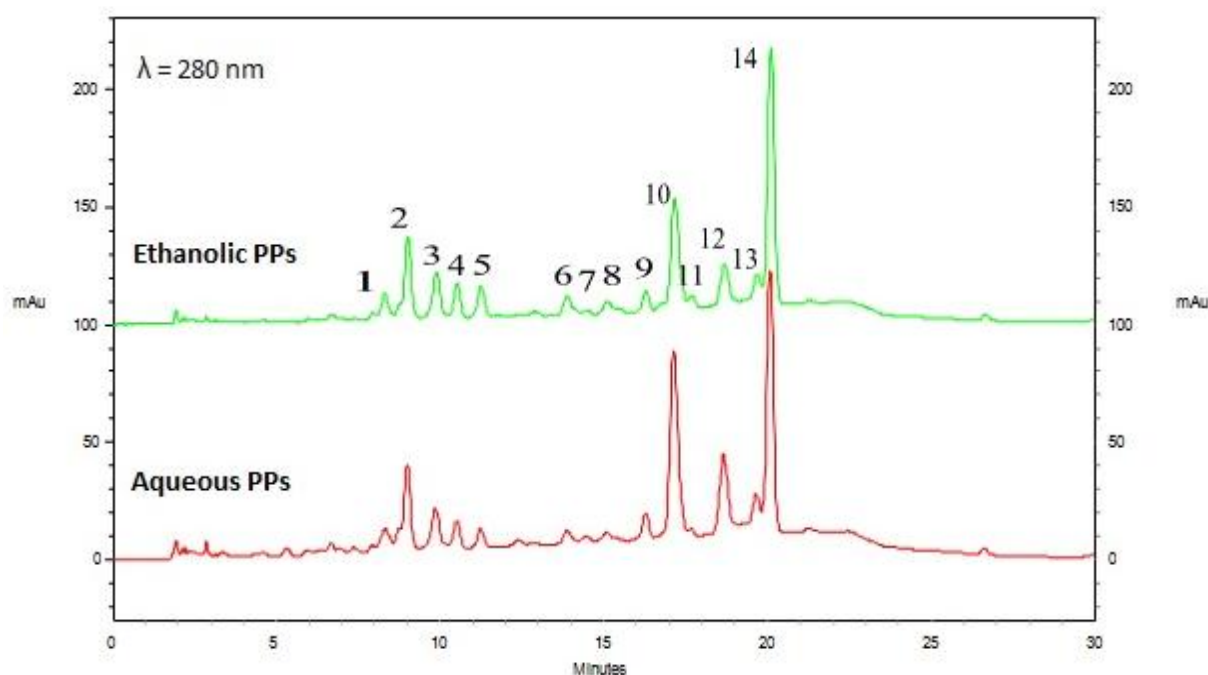


Figure 3.2. HPLC polyphenol profiles ($\lambda=280$ nm) of the ethanolic and aqueous apple skin polyphenolic extracts (from bottom to top). Peak 1 Catechin, Peak 2 Caffeic acid, Peak 3 Chlorogenic acid, Peak 4 Epicatechin, Peak 5 *p*-Coumaric acid, Peak 6 Procyanidin trimer, Peak 7 *m*-Coumaric acid, Peak 8 Ferulic acid, Peak 9 *o*-Coumaric acid, Peak 10 Rutin, Peak 11 Phloridzin, Peak 12 Quercetin, Peak 13 Quercetin derivative, 14 Naringin (Internal standard used). Y axis = mAu (milliabsorbance units)

As highlighted earlier, the PP preparation methods employed have been reported to confer a considerable effect on the final concentration of PPs retained in the sample (Sun-Waterhouse et al., 2011b). This was reflected by the higher concentrations of individual PPs retained in the aqueous extract compared with that of the ethanolic extract (Table 3.2). Apple skin extract prepared using the aqueous method was determined to contain significantly ($P<0.05$) higher amounts of the majority of individual polyphenol compounds (caffeic acid, procyanidin trimer, *m*-coumaric acid, *o*-coumaric acid, rutin, phloridzin, quercetin and its derivative). This agrees with the higher TEPC values in the aqueous extract determined using the Folin-Ciocalteu assay. The amounts of *p*-coumaric acid and ferulic acid detected, however, were marginally higher in ethanolic extract but were statistically insignificant ($P>0.05$). Peak 1 appearing at the front of the HPLC profile (figure 3.2) indicates a molecule of high polarity being eluted at a shorter retention time (in reverse-phase HPLC). Catechin being eluted first from both the skin extracts signifies its more polar nature compared with the rest of the peaks

eluted later. However, the concentration of the relatively more polar compound catechin is much lower compared with the less polar compounds like caffeic acid and chlorogenic acid eluted at longer retention times.

PPs with several hydroxyl groups, such as glycosides are more hydrophilic than the aglycone forms and are more soluble in water or hydroalcoholic mixtures than in pure solvents (Alonso-Salces et al., 2001, Markham, 1982). This is evident from the higher concentration of glycosides like rutin and phloridzin being eluted in our study using the aqueous extraction method compared with that prepared using ethanolic extraction. Glycosides have been previously reported to demonstrate higher solubility in water than their corresponding aglycones (Escribano-Bailon and Santos-Buelga, 2003). However, Alonso-Salces et al. (2001) observed that catechins and hydrocinnamic acids were better extracted using hydroalcoholic mixtures compared with 100% methanol. They further concluded that since catechins and hydrocinnamic acids are relatively more hydrophilic considering their elution earlier than glycosides (in reversed-phase HPLC), water or hydroalcohols are more suitable for their extraction. A similar effect was noticed in our study, wherein higher concentrations of catechin and some hydrocinnamic acids including *m*- and *o*-coumaric acids were detected in the aqueous skin extract, compared with that in ethanolic extract.

Table 3.2. Individual polyphenol species of apple skin extracts obtained from ethanolic and aqueous methods

Polyphenols	Relative amount of individual polyphenol ($\mu\text{g/g}$ extract)	
	Ethanolic PP extract	Aqueous PP extract
Catechin	551.719 ± 7.48^a	590.296 ± 0.55^a
Caffeic acid	1477.62 ± 4.31^a	1603.42 ± 7.13^b
Chlorogenic acid	961.425 ± 2.32^a	1039.87 ± 9.69^a
Epicatechin	656.650 ± 6.91^a	648.245 ± 2.10^a
<i>p</i> -Coumaric acid	712.428 ± 1.50^a	677.097 ± 5.06^a
Procyanidin trimer	725.600 ± 5.38^a	931.313 ± 9.69^b
<i>m</i> -Coumaric acid	339.247 ± 6.11^a	509.749 ± 4.32^b
Ferulic acid	547.974 ± 3.86^a	530.099 ± 2.79^a
<i>o</i> -Coumaric acid	1003.12 ± 8.48^a	1088.03 ± 1.52^b
Rutin	3336.24 ± 2.57^a	5323.81 ± 1.08^b
Phloridzin	466.603 ± 3.78^a	503.346 ± 9.47^b
Quercetin	2242.66 ± 6.22^a	2765.85 ± 2.41^b
Quercetin derivative	1231.19 ± 7.01^a	1665.36 ± 7.33^b

Data are expressed as mean \pm standard deviation. Different lowercase superscript letters (within the same row) indicate statistically significant differences at $P < 0.05$

The amount of glycoside like rutin, extracted from the apple skin using the aqueous method was higher than the aglycone form quercetin. Also, these glycosides (rutin and phloridzin) were retained at higher concentration in the final extract prepared using aqueous methods compared with ethanolic extraction. Overall, rutin, present in both the apple skin PP extracts analysed, appeared to be the largest class present among the individual polyphenolic compounds detected, followed by quercetin and its derivative. Flavonols such as rutin and

quercetin were also amongst the highest detected polyphenols in the apple peels used by McCann et al. (2007). Rutin and quercetin have also been reported to occur at higher levels in apple skin compared with juice and fibre (Sun-Waterhouse et al., 2011b), and thus making apple skin an important dietary source of these flavonols. Rutin and quercetin have been determined to exhibit anti-inflammatory activity in chronic and acute phases (Guardia et al., 2001) as well as confer protection against cardiovascular diseases and cancer due to its high antioxidant activity (AA) (Middleton et al., 2000). Even though only a small amount of phloridzin was detected in the skin extracts, it is important to note that the hydrochalcone glucoside, phloridzin occur exclusively in apple and apple products (Herrmann, 1990). Phloridzin from apple pomace has been shown to possess high antioxidant and radical scavenging activities (Lu and Foo, 2000), thus making it a vital natural antioxidant.

3.3.2 Determination of total antioxidant activity and vitamin C content of apple skin extracts

The antioxidant activity of polyphenols has been suggested to provide protection against various chronic conditions, including lung cancer, cardiovascular diseases and thrombotic stroke (Sun et al., 2002, Wolfe et al., 2003). Similar to the trend of TEPC, the aqueous apple skin extract of this study had significantly ($P < 0.05$) higher TAA (25.69 ± 0.10 mg Trolox eq./g dried extract), than that prepared using the ethanolic method (23.98 ± 0.12 mg Trolox eq./g dried extract) (Table 3.3). The apple fibres prepared using the aqueous method have previously been shown to possess higher TAA than the fibres prepared using ethanolic extraction (Sun-Waterhouse et al., 2011b). The remarkable TAA activity of aqueous skin PP extract compared with that of ethanolic extract can be associated with the higher TEPC content of this extract. The high TAA of apple skin PP extracts in the current study may be attributed to the individual PPs in the extract and vitamin C content.

Table 3.3. Antioxidant activity and vitamin C content of apple skin extracts prepared using aqueous and ethanolic extraction methods

Analysis	Ethanolic PP extract	Aqueous PP extract
TAA (mg Trolox eq./g extract)	23.98 ± 0.12 ^a	25.69 ± 0.10 ^b
Vitamin C (mg/g extract)	0.813 ± 0.28 ^a	0.751 ± 0.33 ^a

Data are expressed as mean ± standard deviation. Different lowercase superscript letters (within the same row) indicate statistically significant differences at $P < 0.05$

The aqueous and ethanolic PP extract contained similar amounts of vitamin C, ranging from 0.751 to 0.813 mg/g extract respectively (Table 3.3). The employment of different extraction methods (ethanolic and aqueous) did not have significant ($P > 0.05$) impact on the amount of vitamin C in the two apple skin extracts. Vitamin C acts as an inhibitor to PP degradation or oxidation, thus making the fruit derived products less susceptible to enzymatic browning (Almeida and Nogueira, 1995, Soliva et al., 2000). It cannot be ignored that even though apple contains some vitamin C (mean value reported is 12.8 mg/100g of apples), Lee et al. (2003) reported that it only contributes towards 11% of the TAA observed. They also determined that the polyphenols occurring in the apples contributed to over 80% of the TAA of the cultivars employed. The apple peels have been reported to exhibit higher TAA than the flesh (Wolfe et al., 2003). The peels of Rome Beauty apples in their study were noted to have TAA of 228.4 ± 6.7 µmol of vitamin C eq/g of peels compared with TAA of 131.6 ± 0.8 µmol of vitamin C eq/g of flesh + peel. This suggests that apple skin is a rich antioxidant source compared with the flesh. And hence, it can be a useful strategy to include the polyphenol extracts from apple skin with high TAA in our diet.

3.3.3 Determination uronic acid content of apple skin extracts

The results of the UA assays revealed that both the ethanolic and aqueous apple skin extracts contained a significant amount of pectic polysaccharides (ranging from 4.45 % to 5.52 %) (Table 3.4). The presence of soluble fibre pectin increases the nutritional value of the

two apple skin extracts. The difference in the amounts of pectic polysaccharides that were present in the aqueous and ethanolic extracts was marginal ($P>0.05$), with ethanolic extract having slightly lower UA content compared with that of aqueous PP extract. The aqueous apple fibre preparation method has been reported to produce fibres with higher UA content than that produced by ethanolic methods (Sun-Waterhouse et al., 2008a, Sun-Waterhouse et al., 2011b).

Table 3.4. Uronic acid content of apple skin extracts prepared using the aqueous and ethanolic extraction methods

Analysis	Ethanolic PP extract	Aqueous PP extract
UA (GalA, %)	4.45 ± 0.47^a	5.52 ± 0.27^a

Data are expressed as mean \pm standard deviation. Different lowercase superscript letters (within the same row) indicate statistically significant differences at $P<0.05$

Pectic polysaccharides, also termed ‘pectins’, are one of the vital dietary fibre components and have gained a lot of interest to be utilized as food additives or functional ingredients (Savary et al., 2003). Pectic polysaccharides are distributed in the middle lamella and primary cell walls of various plant tissues. They occur as complex group of heteropolysaccharides with a backbone of galacturonic acid, ranging from 15 to 25 g/100g of tissues (Sun-Waterhouse et al., 2008a). Pectins have been examined to play important nutritional roles in GI tract in relation to the metabolism of lipids, glucose, minerals and vitamins (Fernandez et al., 2001). Moreover, apple cell wall preparations with high UA content were found to provide greater protection against ascorbic acid degradation (Sun-Waterhouse et al., 2008b). The pectins have the ability to stabilise ascorbic acid. Thus, in addition to providing physiological benefits to humans, apple skin PP extracts produced in our study, which contained pectic polysaccharides, may exhibit some properties of hydrocolloids during food formulation and processing (Sun-Waterhouse et al., 2008a).

3.3.4 TEPC analysis of apple skin polyphenol extract-enhanced milk drinks

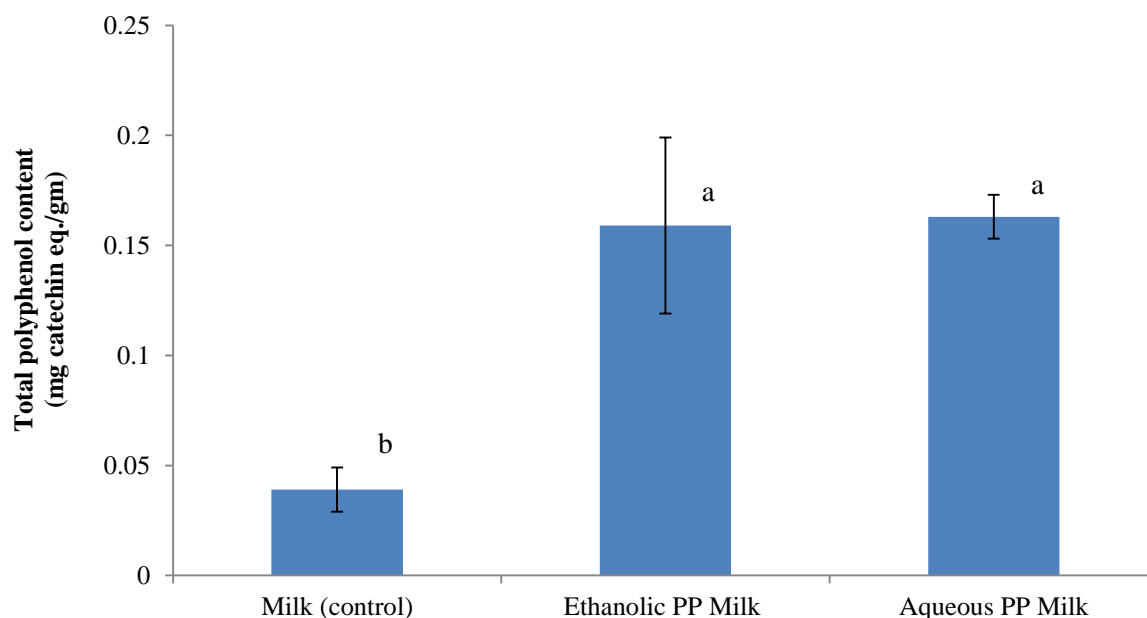


Figure 3.3. Total extractable polyphenol content of control (milk) and apple skin polyphenol extract-enhanced milk drinks. Error bars are the standard deviation of the mean. Different letters indicate statistically significant differences at $P < 0.05$

The significant increase ($P < 0.05$) of TEPC values in the PP-enhanced milk compared with the control milk (as shown in figure 3.3) suggests the retention of added PPs (from the apple extracts) in the fortified milk drinks. The difference in TEPC of the milk drinks enhanced with ethanolic or aqueous PP extract and were statistically insignificant ($P > 0.05$). The TEPC content of control milk drinks were significantly different ($P < 0.05$) from the polyphenol extract-enhanced milk drinks. One gram of each ethanolic and aqueous skin extracts were added to 100 mL milk drinks carrying polyphenol content of 20.66 and 21.44 mg catechin eq./g extract respectively. The TEPC content of milk drinks was noted to be 0.039, 0.159 and 0.163 mg catechin eq./mL milk for control, ethanolic PP milk and aqueous PP milk respectively. The actual TEPC values detected in polyphenol skin extract-enhanced milk drinks were 76.3 % and 76.0% of the theoretical values of the total apple skin PP extracts added to ethanolic milk drink or aqueous milk drinks respectively (as shown in table 3.5). These results suggest that PPs from

apple skin extracts were largely retained after production of milk drinks. In a study by Singh et al. (2012), in which aqueous strawberry extract was added to stirred dahi, 66% of the polyphenol extract was retained after the production of gel matrix. Thirty four per cent of the theoretical value of aqueous strawberry PP extract was detected in the whey portion of dahi. Sun-Waterhouse et al. (2011e) suggested the addition of apple polyphenol extract to yoghurt after fermentation in order to retain higher amounts of the PPs added. Sixty per cent of the theoretical value of the total polyphenol extract added was detected in the low molecular pectin yoghurt after production in their study.

Table 3.5. Percentage of recovery of polyphenols from milk drinks enhanced with apple skin polyphenol extracts

Analysis	Ethanollic PP extract	Aqueous PP extract
PP recovery (%)	76.3 ^a	76.0 ^a

Organic solvents like hexane, acetone and methanol has been successfully applied to extract PPs from various food matrices manually or automatically (Bravo, 1998, Murphy et al., 2002, Richter et al., 1996, Sun-Waterhouse et al., 2009b). Naczk and Shahidi (2004) commented that PP extraction is greatly influenced by differences in the polarity of solvents, type of PPs, and coexisting food components in the food systems. In the current study, a portion of the added PPs might degrade into non-phenolic compounds as a result of heat treatment. The remaining PPs and their derived polyphenolic products might be protected by the milk during processing eg., via binding to milk peptides (Papadopoulou and Frazier, 2004) or by formation of complexes with caseins or whey proteins (Sun-Waterhouse et al., 2011e). Thus, the detected TEPC values were the net results of all these variables. PPs have been reported to bind to proteins, resulting in the formation of soluble or insoluble protein-polyphenol complexes (Richard et al., 2006). The extent of the binding and complexation determines the extractability of PPs and requires suitable solvents for their extraction. The polarity of the skin polyphenolic crude extracts, along with other factors such as pH and structures of the individual PPs as well as their interaction with milk proteins, may also influence their extraction from the milk (Hagerman and Butler, 1981, Singh et al., 2012).

Therefore, the retention of PPs detected in this study might be due to the polarity and the structure of the PP species in the extract as well as their complexation with milk proteins. Glycoside rutin for instance, detected in the crude extract of the apple skin, may be more easily extracted than their aglycone form quercetin. Moreover, the sugar components of the glycosidic PPs may account for TEPC values. A small portion of the TEPC results may come from proteins (e.g., Tyrosine residues) and sugar components (oligosaccharide and glucose) present in the milk powder (Lowry et al., 1951, Singleton et al., 1999, Sun-Waterhouse et al., 2011e), which is evident by the non-zero TEPC reading of the control milk drinks.

3.4 Conclusions

Results obtained from Folin-Ciocalteu assay and HPLC analysis indicated that apple skin is a rich source of PPs. The PP-containing apple skin extract possesses high antioxidant activity as well as have considerable quantities of vitamin C and pectic polysaccharide fibres. These bioactives are known to play vital physiological and nutritional roles in human body.

The chemical analysis also revealed that the preparation methods of apple skin extract significantly influences the amount of TEPC in the final extracts. The aqueous extraction method tends to produce polyphenol extract with high TEPC and TAA relative to ethanolic extraction methods. The higher TAA of PPs has been primarily associated with protective effects against several degenerative conditions (Wolfe et al., 2003). From this viewpoint, the polyphenolic extracts prepared by the aqueous method may be more beneficial than those obtained by ethanolic extraction. Moreover, the aqueous method that utilises water to extract PPs from the skin is more cost-effective, as well as safe and environmental-friendly from industrial perspective. The milk drinks enhanced with apple skin extracts were shown to contain substantial amounts of TEPC. This suggested retention of significant amounts of added PPs in the milk after its production.

The high polyphenolic content and TAA of apple skin extracts prepared in this study indicate that apple skin is a valuable source of polyphenol antioxidants. Hence, it can be used as a value-added food ingredient for products containing probiotic bacteria (PB) in order to provide more favourable conditions for their survival and bioactivity. The next chapter explores the utilisation of apple skin PP extracts as bioactive ingredients for influencing the viability and adhesion of PB.

Chapter 4

Effects of apple skin polyphenols on the viability and adhesion of probiotic bacteria

4.1 Introduction

As discussed in Chapter 2, survival and increased growth of probiotic bacteria (PB) during food processing, storage, handling and digestion as well as their efficient adhesion to intestinal mucosa are desired physiological properties (Stamatova and Meurman, 2009). Dairy products have emerged as potential delivery systems of PB that can carry these beneficial organisms to the consumers. Factors like acidity of matrix environment and oxidative stress to which live PB are exposed, influence their viability. Even though non-viable PB especially heat-inactivated or lyophilised cells have been shown to confer some health benefits in prevention of diarrhoea their effectiveness as well as non-viable status is still questioned (Kaila et al., 1995, Clements et al., 1983). In both these studies viable probiotic cells have been demonstrated to improve diarrhoea significantly than the non-viable cells. The studies also indicate that the heat-inactivated or lyophilised probiotic products cannot be used as controls without verifying their lack of activity.

Low viability of PB could affect their adhesion in the GI tract and thus, may lower the effect of the desired health benefits (Ouwehand and Salminen, 2003, Ouwehand et al., 2002). Efficient adhesion of PB to the GI tract facilitates persistence of PB, enhances healing of damaged mucosa, and eliminates pathogens by competitive exclusion (Vesterlund et al., 2005). The enhanced adhesion of probiotic bacteria such as *L. acidophilus* in the gut promotes resistance to pathogens via competition for intestinal binding sites (Bernet et al., 1994) and production of antimicrobial compounds (Conway, 1996). *L. acidophilus* offers protection against gastrointestinal disturbances that might develop into ulcerative colitis and colon cancer (Wollowski et al., 2001). Hence, improving the adhesion of probiotic bacteria may facilitate the restoration of beneficial microbial flora in the host (Plummer et al., 2005).

PPs have been shown to impart varied effects on the colonic bacteria as well as PB. These effects can either be stimulatory, neutral or inhibitory. For instance, commercial apple PP extract enhanced the growth of *Streptococcus* and *Lactobacillus* in drinking yoghurt, while chlorogenic acid substantially reduced the growth of *Lactobacillus* (Sun-Waterhouse et al., 2011e). In their study, phlorizin exerted minimal change in the growth of *Streptococcus*, but caused reduction in the *Lactobacillus* population. In another study, catechin (100-400 μ M) and chlorogenic acid (400 μ M) were demonstrated to stimulate the growth of *L. casei*, while quercetin (25-50 μ M) caused significant inhibition of PB. Thus, the influence of PPs on PB is governed by the concentration and structure of PPs used and probiotic strain tested. Hence, very low levels (1mg per 100 mL) of pure polyphenol compounds were used in the current study to avoid any inhibitory effect on the growth of *L. acidophilus*. The apple skin PP extracts (1 or 2 % w/v) and the pure PP compounds used in this study appear to be at safe levels of consumption when compared with similar studies (Sun-Waterhouse et al., 2010a, Sun-Waterhouse et al., 2010b, Sun-Waterhouse et al., 2011d, Sun-Waterhouse et al., 2011e). Research by Koren et al. (2009) determined the ability of PPs to bind to the bacterial surface and remarkably improve their total oxidant-scavenging capacities that may provide enhanced protection to mammalian cells against oxygen tension generated during infections and inflammation. This mechanism can also be employed to reduce oxidative stress in the food matrix in order to provide a more suitable environment for the survival of PB. Moreover, the ability of PPs to influence the growth and adhesion of gut bacteria to colonic cells has been investigated (Parkar et al., 2008). The synergistic combination of PPs and probiotics may provide an opportunity to produce cells with enhanced attachment ability and tolerance to environmental stresses.

The spread plate method was used to evaluate the viability of probiotic *L. acidophilus* using deMan, Rogosa & Sharpe (MRS) agar. This selective medium allows the growth and enumeration of *Lactobacillus* cultures in dairy and other food products (De Man et al., 1960). The plating method presents advantages, such as ease of preparation, cost-effectiveness and measurement of live bacteria only. The one drawback of using this method is the long waiting period (24-48 h) before the results can be obtained. Crystal violet (CV) attachment assay has been used to investigate the adhesion capacity of probiotic *L. reuteri*, *L. rhamnosus* GG, *Roseobacter* Strain 27-4 (Bruhn et al., 2006, Jones and Versalovic, 2009, Lebeer et al., 2007).

The CV assay commonly finds application in determination and quantification of bacterial biofilms and normally uses 96-well polystyrene microtitre plates as the attachment surface (Oh et al., 2007). Previous *in vitro* studies aimed at examining the adhesion of probiotic bacteria to the GI tract utilized models including intestinal mucus (Collado et al., 2005, Matsumoto et al., 2002) and tissue culture cells (Aissi et al., 2001, Parkar et al., 2008). The use of tissue culture cells has a potential disadvantage, since these cells are cancer cells which may or may not be different from normal intestinal epithelial cells (Ouwehand and Salminen, 2003, Rousset, 1986). In our study, hydrophilic tissue culture treated plates were employed as a model surface to reliably investigate the effect of PPs on the adhesion capacity of PB. It is acknowledged that this method does not provide the normal physiological attachment sites and conditions of the intestinal epithelium. The adhesion was investigated under aerobic and anaerobic conditions at two temperatures: 37 °C and 4 °C. The body temperature (37 °C) was chosen to provide realistic and physiological conditions occurring in the intestine while, cold temperature (4 °C) provides the picture of adhesion of PB in the oral cavity soon after consumption of cold milk drinks. It is acknowledged, however, that the contact time of the probiotic milk in the oral cavity is much shorter than 3 hours when drinks are actually being consumed. The 3-hour incubation time was chosen in this study for ease of collecting significant data.

4.2 Materials and Methods

4.2.1 Chemicals and materials

The chemicals and materials used are listed in Table 4.1. The glassware and materials used for microbiological assays were sterilised by autoclaving at 121 °C for 20 min. The viability and attachment assays were performed in sterile conditions in a laminar flow workstation (EMAIL AIR HANDLING cabinet, AES environmental Pty Ltd. NSW, Australia).

Table 4.1 Chemicals and materials used in this study

Chemicals and Materials	Manufacturer
<i>Lactobacillus acidophilus</i>	Obtained from AUT culture collection (Courtesy of Dr. Noemi Gutierrez-Maddox)
deMan, Rogosa & Sharpe (MRS) broth and agar	Difco Laboratories Inc, Detroit, Michigan
Peptone extract	Bacto™ Peptone, BD, Difco , NSW, Australia
Methanol, glycerol and glacial acetic acid	Ajax Finechem Ltd, Sydney, Australia
<i>p</i> -coumaric acid, chlorogenic acid, rutin, epicatechin, quercetin, phlorizin and Folin-Ciocalteu phenol reagent	Sigma-Aldrich, St Louis, MO, USA
Crystal violet	Hopkins & Williams Ltd, Chadwell Heath Essex, England
Instant Skim milk powder	Pam's, New Zealand. Batch no: B2816062, purchased from New World supermarket, Auckland

4.2.2 Preparation and analyses of polyphenol-enhanced probiotic milk drinks

4.2.2.1 Preparation of apple skin polyphenol extracts

The apple skin polyphenol extracts were prepared by either the ethanol or aqueous extraction methods as previously described in Chapter 3. Hence, all the chemicals and materials used in the PP extract preparation are the same as described under section 3.2.1.

4.2.2.2 Microbial growth conditions and maintenance

The probiotic culture, *L. acidophilus* was obtained from fresh cultures on de Man, Rogosa & Sharpe (MRS) agar. A few colonies from the MRS agar were aseptically transferred, using an inoculating loop, into glass vials containing 10 mL sterilised MRS broth, and then incubated at 37 °C for 24 h in an atmosphere with 5% CO₂ in air (henceforth referred to as an anaerobic incubation). An aliquot (0.1 mL) of overnight culture was spread plated onto MRS agar medium in Petri plates, and incubated anaerobically at 37 °C in a CO₂ incubator (Sanyo Electric Co., Ltd, North America, USA) for 48 h to allow lawn growth. Cotton swabs were used to transfer bacteria from the MRS agar medium to sterile MRS broth containing 15% glycerol. The working and museum cultures of *L. acidophilus* were stored frozen in MRS broth containing 15% glycerol at -20 °C and -80 °C, respectively until required. The working cultures stored at -20 °C were used for this study and were thawed before use. The museum cultures prepared from the original culture were maintained at -80 °C for long term storage.

4.2.2.3 Preparation of probiotic cell suspension

The inocula for experiments with the probiotic organism *Lactobacillus acidophilus* were obtained after thawing the frozen working cultures. An aliquot (10 mL) of sterile MRS broth in a glass vial was inoculated with 1 mL bacterial culture. These glass vials were then placed in a CO₂ incubator to allow anaerobic incubation at 37 °C for 24 h. The bacteria were transferred twice successively in MRS broth and subjected to anaerobic incubation at 37 °C. After 24 h, the bacteria were harvested by centrifugation (Eppendorf centrifuge 5810R, Eppendorf, Hamburg, Germany) at 4000 rpm at 4 °C for 10 min. The supernatant was discarded, and the cell precipitate was collected and washed thoroughly with sterile 0.1% (w/v) peptone water. This washing procedure was repeated three times, and the cell slurry was collected and topped up to 10 mL by adding the required quantity of sterilised peptone water (termed “PB culture suspension”).

4.2.2.4 Preparation of polyphenol- and probiotic-enhanced milk drinks

Skim milk (12% w/v) was prepared by dissolving a desired amount of skim milk powder in filtered RO water and homogenising using a Silverson mixer (X screen, Silverson Machines Inc., East Longmeadow, MA, USA) at 2000 rpm for 3 min. Polyphenol- enhanced milks were prepared in duplicate for each formulation by adding either ethanolic or aqueous PP extracts to skim milk at 1% (w/v) or 2% (w/v) concentration. The formulations were gently mixed using a Silverson mixer at 1000 rpm for 2 min, and then subjected to heat treatment at 85 °C for 30 min using the double boiler technique. The milk drinks were then allowed to cool to approximately 35 °C. Six commercially available, purified pure polyphenol compounds: *p*-coumaric acid, chlorogenic acid, rutin, epicatechin, quercetin and phlorizin were also used to prepare “spiked” milk drinks to evaluate their effect on the viability of probiotic bacteria, each PP was added to the skim milk at the rate of 1 mg PP per 100 mL milk. The spiked milk drinks formulated with purified PP compounds were also subjected to heat treatment at 85 °C for 30 min, using the double boiler technique. The spiked milk drinks were then allowed to cool to approximately 35 °C.

The PB culture suspension was thoroughly mixed using a vortex mixer, and an aliquot (1 mL) was inoculated to the PP-enhanced milk drinks (appendix IV). Skim milk (100 mL) inoculated with 1 mL *L. acidophilus* culture suspension in the absence of added polyphenols was set up as a control (termed as “control PB milk”). The milk bottles were wrapped with aluminum foil to avoid interference of light with the polyphenols and were stored at 4 °C for 50 days.

4.2.2.5 Enumeration and viability of probiotic bacteria in polyphenol-enhanced milk drinks

The bacterial enumeration for estimating the viability of *L. acidophilus* in polyphenol-enhanced milk drinks was performed on sub-samples (1 mL) withdrawn from the treated and control PB milk drinks at intervals of 5 days from Day 0 to Day 50. Each 1 mL milk sub-sample was diluted with 9 mL of 0.1% (w/v) peptone water, and mixed uniformly with a vortex mixer. Serial 10-fold dilutions with sterile peptone water were plated in triplicate onto

MRS agar plates, using an automatic spiral plater (Don Whitley Scientific Limited, West Yorkshire, England). The plates were then incubated at 37 °C anaerobically for 48 h in a CO₂ incubator. The viable numbers of *L. acidophilus* were expressed as Log CFU/mL and calculated using the following formula:

$$\text{Viable number} = \text{Log} \frac{\text{Number of CFU}}{\text{Volume plated (mL)} \times \text{total dilution used}}$$

4.2.3 Determination of influence of polyphenols on the adhesion of probiotic bacteria

4.2.3.1 Crystal violet attachment assay

The crystal violet (CV) attachment assay used in this study was based on the method of Oh et al. (2007). The surface of 24-well flat-bottomed tissue culture treated polystyrene microtitre plates (BD Falcon™, BD Biosciences, NSW, Australia) was used for the attachment assay because they provide a hydrophilic surface for bacterial attachment and larger surface area than the normal 96-well plates. Polyphenolic-enriched MRS broth was prepared by adding 1 g of either aqueous or ethanolic PP extract to 100 mL of sterilised MRS broth while the broth was still hot (approximately 70 °C). Complete dissolution and subsequent cooling to room temperature of the PP extracts was facilitated through stirring using a magnetic stirrer. Aliquots (1800 µL) of the resulting polyphenol-enriched MRS were added to the wells of microtitre plates. Overnight culture of *L. acidophilus* (200 µL) was added to each well, and the plates were incubated aerobically or anaerobically at 37 °C or 4 °C for 3 h. MRS broth inoculated with *L. acidophilus* culture in the absence of added polyphenols was set up as the control (termed “Control PB MRS”). Six commercially available purified PP compounds : *p*-coumaric acid, chlorogenic acid, rutin, epicatechin, quercetin and phlorizin were also used (1 mg each) in the same way to examine their effect on the adhesion of PB. Sterile MRS broth (2000 µL) was included as a blank.

The contents in the wells of the plates were removed after incubation, and the emptied wells were washed with 2500 µL sterilised deionised water. The adherent cells were fixed with 2000 µL of 96% methanol per well for 15 min. After the methanol was discarded, the plates were air-dried. The wells were stained with 2000 µL of crystal violet (0.5% (w/v)) for 5 min. After the stain was rinsed off under a gentle stream of running deionised water, the plates air-dried. The dye was extracted from the attached cells with 2000 µL of 33% (v/v) glacial acetic acid per well. The optical density (OD) of each well was measured at 595 nm using the microtitre plate reader (BMG Labtech™, FLUOstar omega Microplate reader, Imgen Technologies, USA).

4.2.4 Statistical analysis

Experimental data were statistically analysed using Minitab® (version 16) software (Minitab Inc., State College, Pennsylvania, USA) with Analysis of Variance (ANOVA), using a repeated measures design for viability and adhesion assays. At least two observations per analysis were performed and the results were expressed as mean values and standard deviation of the mean. The difference is considered to be of significant importance at more than 95% confidence interval ($P < 0.05$). When significant differences occurred, Tukey's HSD (Honestly significant difference) test was employed to examine where that difference occurred.

4.3 Results and Discussion

4.3.1 Viability of probiotic bacteria in polyphenol-enhanced milk drinks

Overall, the skin PP extracts at 1% and 2% concentrations had similar effects on the viability of *L. acidophilus* during the 50-day storage trial (Figure 4.1 and 4.2), exerting significant protection ($P < 0.05$) on *L. acidophilus* in the skim milk matrix compared with the control PB milk. As depicted in figure 4.1 and 4.2, the number of *L. acidophilus* cells in the control milk drinks declined gradually from Day 0 till Day 30, followed by a rapid decrease to below the required minimum level (10^6 CFU/mL) towards the end of storage trial. Such a decrease in the number of *L. acidophilus* indicated the inability of this PB to maintain a constant viability in the control milk (i.e. in the absence of added PPs). Similar observations for control milk were found in previous studies (Iwana et al., 1993, Shah et al., 1995, Varnam and Sutherland, 1994), including PB like *L. acidophilus* and *Bifidobacterium* in dairy products before the expiry date. Various factors, including product acidity, oxidative stress, permeation of oxygen through the package, exposure of PB to oxygen after opening of the package prior to consumption and lack of nutrients in the milk, have been suggested as accounting for the low viability of PB in the dairy products (Dave and Shah, 1997a, Shah, 2000, Shah et al., 2000, Tamime et al., 1995). PB especially have been reported to display poor growth in milk, owing to lack of appropriate nutrients (Kailasapathy and Chin, 2000). Addition of 0.01% baker's yeast to milk (Shimamura, 1982), and fortification of skim milk with oligosaccharides and inulin (Shin et al., 2000) have been shown to improve the survival of these beneficial organisms, depending on the carbon source and concentration. Loss of viability of PB is reported to be greater in fermented milk, compared with unfermented milk, because of acid injury to the bacteria (Hughes and Hoover, 1995). However, lactose assimilation has been reported as a main limiting factor for growth of lactobacilli in milk (Desai et al., 2004). Thus, incorporation of prebiotics (such as oligosaccharides and inulin) has been suggested for enhancing the growth of PB in milk (Desai et al., 2004, Shin et al., 2000).

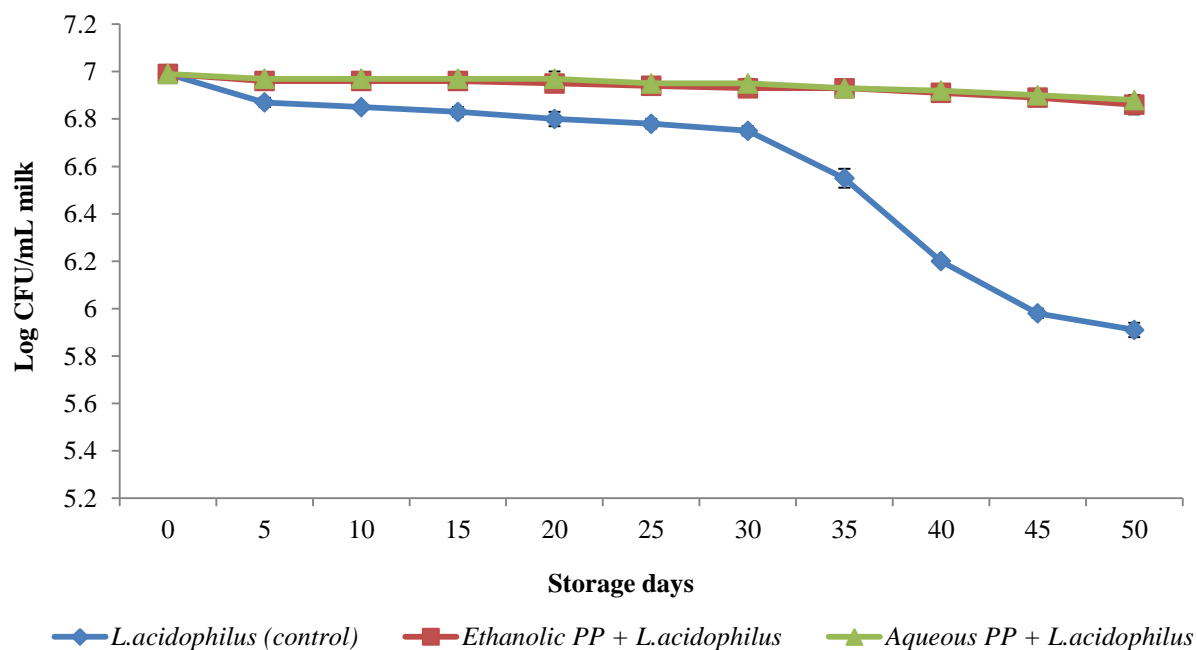


Figure 4.1. Effect of apple skin polyphenol extracts at 1% on the viability of probiotic bacteria in milk drinks

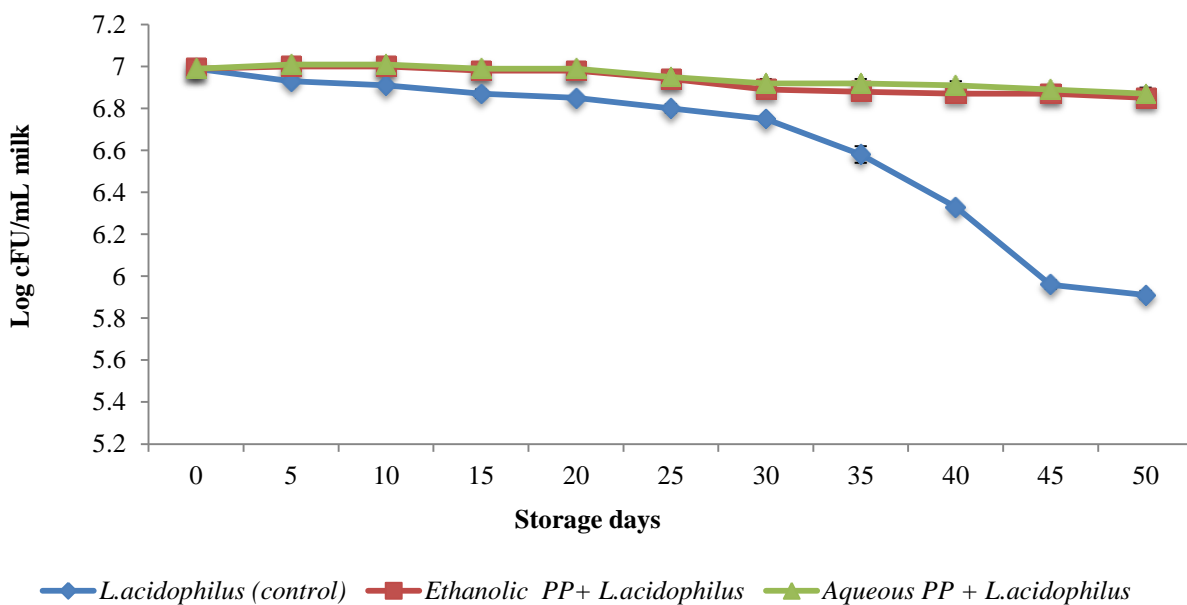


Figure 4.2. Effect of apple skin polyphenol extracts at 2% on the viability of probiotic bacteria in milk drinks

The Tukey's statistical analyses revealed that the two apple skin extracts in maintaining the viability of *L. acidophilus* in the current milk drinks were significantly effective ($P < 0.05$). However, the difference in the efficacy of the two extracts was statistically insignificant ($P > 0.05$), with the aqueous PP extract being only slightly better than the ethanolic PP extract. The results of the study by Sun-Waterhouse et al. (2011e) also reported that the commercial apple PP extract facilitated the proliferation of *Streptococcus* and *Lactobacillus* in drinking yoghurt when added prior to fermentation. The addition of apple polyphenol extracts in their study boosted the growth of both starter cultures in the yoghurt compared with the control. Aqueous extracts of apple, banana and orange were also found to enhance the growth of probiotic *Lactobacillus* and *Bifidobacterium* strains, and inhibit pathogenic bacteria (Sutherland et al., 2009).

Fruit extracts have been previously shown to exert a stimulatory effect on the proliferation of probiotic species in a dose-dependent manner (Sophorn, 2010). The aqueous fruit extracts in their study were prepared by homogenizing the dried fruit pulp in sodium phosphate buffer (pH 7.4). The aqueous extracts of strawberry, blueberry, green kiwifruit and feijoa at high concentration (20-30%) stimulated the growth of probiotic *Bifidobacterium* and *Lactobacillus* species, with the exception of *B. longum*. The higher concentrations of these extracts were found to repress the growth of *B. longum*, while, increased growth was observed at 10% concentration. Strawberry, green kiwifruit and feijoa extracts have been demonstrated to exhibit biphasic effects on the growth of PB, with low concentrations (0.01 g/L to 0.94 g/L) of these extracts inhibiting probiotic growth, while enhancing the growth at high concentration (1.88 g/L to 30 g/L). At high concentration (30 g/L) all these fruit extracts were found to inhibit both Gram-negative and Gram-positive pathogenic bacteria. However, the blueberry and strawberry extracts at the concentration < 30 g/L were found to stimulate the growth of some pathogens, including *Vibrio parahaemolyticus* and *Yersinia enterocolitica*. Green kiwifruit extract at a concentration as low as 3.75 g/L was found to inhibit the proliferation of *V. parahaemolyticus*. In our study, the cell count of *L. acidophilus* from milk drinks enhanced with purified, ethanolic or aqueous apple skin PP extracts remained well above that of control PB milk throughout the storage period. This suggests that the PPs and their concentrations used did not inhibit growth of the *L. acidophilus* strain used.

Moreover, no contamination from mould or Gram-negative bacteria was detected in the milk drinks throughout the storage trial in this study, as confirmed using Gram-staining.

A variety of active compounds, including polyphenols, sugars and proteins present in the aqueous extracts of strawberry, blueberry, green kiwifruit and feijoa, were speculated to stimulate the growth of PB in a study by Sophorn (2010). The precise mechanism by which PPs enhanced the growth and activity of PB is yet to be understood. It is possible that PPs provided additional energy to PB and/or acted as potent antioxidants, ultimately leading to their improved growth (Koren et al., 2009, Parkar et al., 2008, Sophorn, 2010). In our study, the higher polyphenol content and TAA of aqueous apple skin PP extract may have provided greater protection of PB in the milk drinks compared with ethanolic extract, by reducing the oxidative stress. The antioxidant ascorbic acid (vitamin C) was also found to slow down the reduction of *L. acidophilus* counts in yoghurt stored at 4 °C (Dave and Shah, 1997b). The polyphenolic compounds from blueberry extracts are thought to be metabolised during the growth by *Lactobacillus* and *Bifidobacterium* (Molan et al., 2009b) which facilitates greater survival of PB in broth supplemented with the extract.

Among the pure PPs tested (see figure 4.3), the ability of rutin to preserve the viability of probiotic bacteria appeared to be highest. The viability of probiotic *L. acidophilus* in the PP-enhanced milk drinks decreased in the order of rutin > epicatechin > phlorizin > chlorogenic acid > quercetin > *p*-coumaric acid. All the purified PP compounds at the concentration used in this study (1mg/100 mL of milk) reduced the rate of loss of viability of the PB. *p*-Coumaric acid demonstrated the least effectiveness ($P<0.05$) in promoting survival of probiotic bacteria in the milk drinks under the conditions of this current study. In the absence of added PP compounds (the control PB milk), the viability of *L. acidophilus* dropped from 6.99 Log CFU/mL (Day 0) to 5.91 Log CFU/mL (Day 50). Tukey's statistical test revealed that rutin may exert the same effect ($P<0.05$) as the ethanolic apple skin extract on preserving the viability of PB in the milk. A substantial difference in the efficacy of aqueous and ethanolic PP extracts from purified PP compounds may also be attributed to the synergistic effect of total TAA, vitamin C along with soluble fibre content of these crude apple skin extracts.

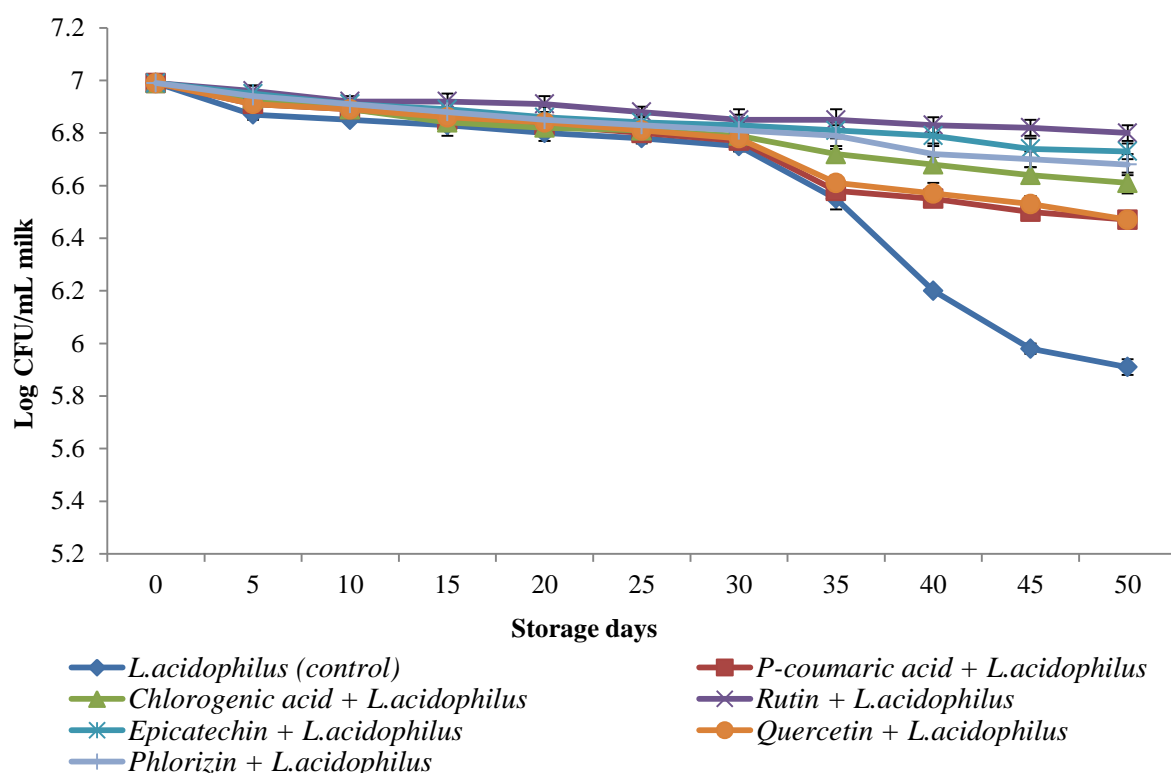


Figure 4.3. Effect of pure polyphenol compounds on the viability of probiotic bacteria in milk drinks

Pure PP compounds have been reported to exert different effects on the probiotic bacteria depending on concentration, structure and probiotic strain used. Chlorogenic acid has been examined by Sun-Waterhouse et al. (2011e) and found to cause slight reduction in the *L. acidophilus* population, while improving the growth of *Streptococcus* in drinking yoghurts. In the same study, phlorizin remarkably negatively affected the growth of *L. acidophilus*. In our study, both chlorogenic acid and phlorizin reduced the loss of the *L. acidophilus* in the milk drinks. Chlorogenic acid and phlorizin at the concentration of 1 mg/100mL of milk appeared to maintain the viability of probiotic bacteria above the required minimum level ($>10^6$ CFU/mL) throughout the storage period. The employment of different concentrations of pure polyphenols and different probiotic strains may explain the discrepancies in the two studies. In another study, catechin, quercetin and chlorogenic acid were found to promote the growth of *L. casei* in a concentration dependent manner (Duda-Chodak et al., 2008).

The role of chemical structure of PPs was obvious with respect to viability of probiotic *L. acidophilus*. For example, rutin, which is a glycoside of quercetin, conferred significantly ($P<0.05$) more protection to probiotic bacteria in the milk than its aglycone form quercetin. This suggests that glycosidic forms of PPs might be more effective in sustaining the viability of PB than their aglycosidic forms, especially in case of flavonoids. Similarly, the glucoside phlorizin was as effective ($P<0.05$) in maintaining the viability of PB as rutin. The glucosides have been reported to be hydrolysed into their respective aglycosidic forms by the intestinal microflora through metabolism (Manach et al., 2004). This partly explains the significant protective effect of rutin on probiotic bacteria in our study as these glycosides serve as a source of energy for probiotic bacteria during metabolism. Hydroxycinnamic acids like the chlorogenic acid and *p*-coumaric acid used in the study, were least effective in sustaining the viability of PB in milk. Another aspect that should be considered is the possibility of transformation of PPs by bacteria as suggested by Duda-Chodak et al. (2008). Catechin and gallic acid have been shown to activate the growth of *L. hilgardii*, possibly by metabolising these PPs to catechol and simple phenols, and their derivatives (Alberto et al., 2001). Therefore, PB may utilise the PPs as a source of energy for metabolism, thereby enhancing their growth and survival.

In addition to the individual PP composition, the AA of PPs may be important in the promotion of viability and growth of probiotic bacteria (Duda-Chodak et al., 2008, Koren et al., 2009, Parkar et al., 2008). Chlorogenic acid and quercetin at more than 100 μM concentration have been shown to exert remarkable stimulation in the growth of *L. casei* (Duda-Chodak et al., 2008). The authors believed that both AA and chemical structure were responsible. The fact that chlorogenic acid had several-times lower AA than catechin, but still exerted a positive effect on PB, and that quercetin, with its higher AA inhibited bacterial growth at an elevated concentration, suggests the interplay of AA value with PP chemical structure. Tea phenolics, such as caffeic acid, demonstrated a strong inhibitory action against the growth of intestinal pathogens including *Escherichia coli*, *Salmonella*, *Pseudomonas*, *Clostridium* and *Bacteroides*, with no inhibitory impact on probiotic microflora, in a study by Lee et al. (2006). Catechin in their study had no effect on *Clostridium*, while enhancing the proliferation of *Lactobacillus* and *Bifidobacterium*. Thus, the maintenance of viability of *L. acidophilus* in the PP-enhanced milk drinks in our study may have been influenced by the

chemical structure of the PPs. These results are in agreement with those observations from studies reported above. The highest concentration of rutin in both the skin extracts (chapter 3, see table 3.2) indicate the significant contribution of rutin to the beneficial efficacy of the two apple skin extracts in preserving the viability of PB in the milk drinks.

4.3.2 Influence of polyphenols on the adhesion of probiotic bacteria

The adhesion of PB to the gastrointestinal cells is a prerequisite for these bacteria to impart the health benefits to the host (Salminen et al., 1996). The efficacy of PPs in mediating the adhesion of probiotic bacteria was evaluated using hydrophilic tissue culture treated microtitre plates in the crystal violet attachment assay. Overall, the adhesion pattern of probiotic *L. acidophilus* followed a similar trend at the two incubation temperatures, 37 °C and 4 °C, though the magnitude of some optical density readings was slightly different under the two conditions (Figure 4.4).

The technique used for the bacterial adhesion assay detects the total bacteria adhering to the hydrophilic surface of microtitre plates. At 37 °C, the optical density (O D) of *L. acidophilus* culture without added polyphenols (Control PB MRS) was 0.12 and 0.24, respectively, for the incubation under aerobic and anaerobic conditions. The aqueous PP extract from apple skin promoted probiotic adhesion to a greater extent ($P < 0.05$) under both aerobic (O D = 0.64) and anaerobic (O D = 0.99) conditions, than did purified PPs and the ethanolic PP extract. At 4 °C, the *L. acidophilus* control culture attachment values were O D 0.11 and 0.24, respectively, for incubation under aerobic and anaerobic conditions. The effect of temperature on adhesion was not statistically significant, which suggests that *L. acidophilus* present in a cold milk drink at 4 °C and drink warmed after consumption could attach to the cells of the oral cavity and later the GI tract. The aqueous apple skin PP extract facilitated strong adhesion of bacteria at 4 °C under aerobic (O D = 0.54) and anaerobic (O D = 0.89) incubation conditions. The ability of PPs to influence the attachment of PB at relatively low temperatures can be utilised to promote attachment of PB in the oral cavity through consumption of refrigerated dairy products such as milk drinks or ice cream. The oral cavity is the primary entry to the GI tract and the ingested probiotics are exposed first to

saliva which mediates the contact with oral tissues (Stamatova and Meurman, 2009). In a previous *in vitro* study, Haukioja et al. (2008) reported that *Lactobacillus* and *Bifidobacterium* strains cannot grow in the saliva but have the ability to remain viable after 24 h of incubation. This may allow sufficient time for PPs to mediate significant attachments of PB to the oral tissues as indicated by the results of our work. Incubation of *L. acidophilus* with PPs for 3 h was observed to promote significant adhesion in the current study.

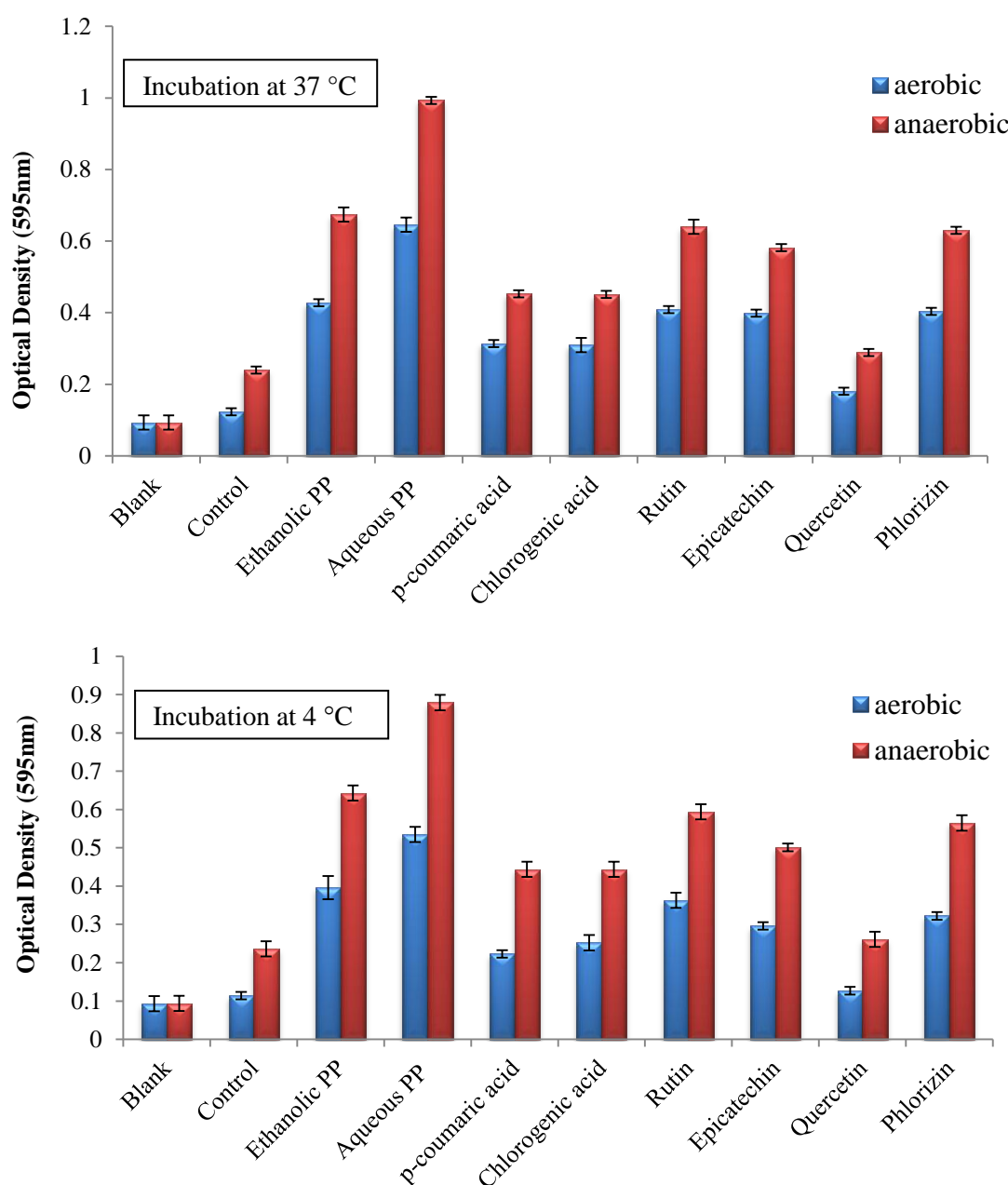


Figure 4.4. Effect of apple skin polyphenol extracts (ethanollic and aqueous) and pure polyphenols on the adhesion of probiotic bacteria at 37 °C and 4 °C

Interestingly, the anaerobic condition at either temperature was found to facilitate greater adhesion of probiotic cells than the aerobic condition. The adhesion of *L. acidophilus* mediated with or without added PPs was significantly higher ($P<0.05$) under anaerobic conditions than under aerobic conditions. The reason behind this result still remains unclear. One possible explanation could be that anaerobic condition allowed the growth of *L. acidophilus* under less oxidative stress, subsequently leading to higher activity and greater adhesion.

At either temperature, the ethanolic PP extract was as effective in promoting adhesion as rutin, which was followed by phlorizin and epicatechin. These results mostly agreed with those found by Parkar et al. (2008) who reported the dose-dependent increase in adhesion of *L. rhamnosus* to colonic adenocarcinoma cells in the presence of rutin and phloridzin in comparison with other PPs that exhibited inhibitory effects. Parkar et al. (2008) also found that dietary PPs suppress the adhesion of pathogenic bacteria. In our study, quercetin was the least effective in improving the adhesion of *L. acidophilus*, which was statistically the same ($P>0.05$) as the control. Chlorogenic acid and *p*-coumaric acid did not facilitate strong adhesion of *L. acidophilus*, compared with the apple skin PP extracts. The O D of PP-enhanced medium containing PB did not fall below that of “MRS PB control”. This indicates that neither the apple skin extract, nor the pure polyphenol compounds tested in the study, imparted any toxic effect on the probiotic culture.

The chemical structure may play an important role in the attachment. The influence of glycosylation of PPs on the adhesion of *L. acidophilus* was found which agreed to the findings observed in the viability assay. A greater enhancement in adhesion was induced by rutin, compared with quercetin. The significantly higher amount of rutin and phloridzin found in the aqueous PP extract may be correlated with its greater adhesion-enhancing effect, compared with the ethanolic PP extract. The mode of action through which PPs stimulate the adhesion to intestinal cells is still not understood. In the study by Huber et al. (2003) catechin and epigallocatechin were reported to have a similar effect to acetylated homoserine lactones, which regulate biofilm formation (the initial step of the bacterial adhesion).

4.4 Conclusions

Results obtained from the viability and attachment assays of this study indicate the potential of PPs to preserve the viability and enhance the adhesion of probiotic *L. acidophilus* to a hydrophilic surface. The results of this study agree with the findings of previous studies by Parkar et al. (2008) and Duda-Chodak et al. (2008) on the positive influence of PPs on the growth and attachment of colonic microflora and PB.

The ability of apple skin PP extracts to preserve the viability of *L. acidophilus* in milk drinks during refrigerated storage confirmed the efficacy of apple skin polyphenols to enhance probiotic activity. Both the aqueous and ethanolic PP extracts at 1 and 2% concentrations showed the same effects on maintaining the viability of the probiotic above the required minimum level ($>10^6$ CFU/mL) over the 50-day storage at 4 °C. Among the purified polyphenols used in this study, rutin was the most powerful PP, maintaining survival of the PB on the same scale as that of the ethanolic skin extract. Epicatechin and phlorizin had the second highest power to preserve probiotic cells in milk drinks during storage at refrigerated conditions. Coumaric acid provided the least protection to PB cells but still was beneficial compared with the control. For the purified PP compounds, the glycosylated forms (such as rutin) might be more effective in maintaining the survival of PB in the milk drinks than their aglycone counterparts. The significantly higher TAA and polyphenol content of aqueous skin extract was reflected by the remarkable ability to provide greater protection to probiotic cells in milk drinks compared with ethanolic skin extract and the purified PP compounds tested. The skim milk in the current study served as a simple medium to precisely determine the effect of PPs on the viability of PB without the interference from fermentation generated compounds found for instance in yoghurt. Moreover, considering that milk was used for viability assay that contained PPs, the covalent binding effect between the milk proteins and PPs (Kroll et al., 2003, Rohn et al., 2004) clearly did not stop the probiotic viability enhancing effect of PPs.

Similar trend as detected in viability assays were found in the attachment assay. The aqueous apple skin PP extract facilitated a greater attachment of *L. acidophilus* to the hydrophilic plate surface than the ethanolic extract. Significant adhesion of *L. acidophilus*

was found to be mediated by both apple PP extracts and purified PP compounds at both 37 °C and 4 °C incubation temperatures. Rutin demonstrated the greatest capability to promote the adhesion of probiotic cells amongst all the purified PP compounds. Phlorizin and epicatechin had moderate power, while quercetin was least effective in influencing the adhesion of *L. acidophilus* cells.

Importantly, the two apple skin PP extracts or the purified PP compounds exhibited much greater preservation on the *L. acidophilus* at the concentrations used in this study, compared with the control. The PPs were extracted from apple skin waste for use in this study. This aligns with the industrial waste reuse strategy, which suggests the important practical influence of this current study.

In the next chapter, a strategy to maximize the delivery of PPs and PB in the milk drink system through microencapsulation technology was investigated.

Chapter 5

Microencapsulation of probiotic bacteria in the presence and absence of an apple skin polyphenol extract

5.1 Introduction

The work reported in this chapter aimed to explore the efficacy of a microencapsulation technology, co-extrusion, to maintain the viability of probiotic *L. acidophilus* without compromising the delivery of the goodness of apple skin PP extracts.

As reviewed in the Chapter 2, the co-extrusion encapsulation has been used to produce beads to carry bioactives, including PPs (Carr et al., 1991, Sun-Waterhouse et al., 2011c, Sun-Waterhouse et al., 2011d, Yilmaz et al., 2001). Only few studies used the co-extrusion technology to encapsulate PBs, e.g. encapsulation of *L. rhamnosus* (Ying et al., 2007) and *L. acidophilus* (Iyer and Kailasapathy, 2005, Jankowski et al., 1997). The resultant encapsulated probiotic cells have demonstrated relatively high viability, owing to minimal cell injury and biocompatibility (Iyer and Kailasapathy, 2005, Jankowski et al., 1997, Mortazavian et al., 2007). In our study, the co-extrusion technique was employed to co-encapsulate probiotic *L. acidophilus* cells with aqueous and ethanolic apple skin PP extracts. Alginate was used as the encapsulant material for the shell of microbeads generated using co-extrusion technology, and as the medium component for the core solution of microbeads. Such approaches to utilise alginate polymer for co-extrusion encapsulation have been previously reported, owing to the harmlessness of alginate towards PB, ease of handling and low cost of alginate (Sultana et al., 2000, Sun-Waterhouse et al., 2011c, Sun-Waterhouse et al., 2011d).

The encapsulated beads generated in the study were examined, using optical and environmental scanning electron microscopy (ESEM), in terms of their shape, morphology, size and wall thickness (Sun-Waterhouse et al., 2011c). Cryo-SEM was employed to show the microstructure of encapsulated beads and the existence of PB inside the beads (Allan-Wojtas et

al., 2008). The microencapsulation efficiency PB was calculated to evaluate the success of the microencapsulation technique of this study. Furthermore, TEPC and TAA of the beads with apple skin PP extract were determined to examine the preservative effect of the co-extrusion encapsulation method on apple skin PP antioxidants. Such efficacy evaluations were performed at 50 days and under refrigerated conditions.

The beads carrying probiotic cells were exposed to an acidic solution (pH 2) for 120 min to explore the integrity of the alginate wall and the tolerance of encapsulated PB under acidic conditions, to simulate the pH of the stomach and thus the conditions encountered by PB in the upper digestive tract. Other workers have used various incubation times and pH values in testing survival of probiotic bacteria under acidic conditions mimicking the gastric environment *in vitro*. Studies by Heidebach et al. (2009) and Reid et al. (2005) reported an incubation time of 90 min, while others tested the survival of bacteria in acidic conditions for 120 min (Annan et al., 2007, Borges et al., 2012, Ding and Shah, 2007, Ding and Shah, 2009, Podolsky, 1991). The survival of probiotic cells in acidic conditions has also been examined for up to 3 h (Iyer and Kailasapathy, 2005, Mandal et al., 2005). Owing to the constantly changing environment in the gastric region the precise acidity is difficult to ascertain but is considered to vary between pH 1.5 and 3.5. Martoni et al. (2007) investigated the nature of *L. plantarum* 80 cells using a dynamic computer-controlled model simulating the human GI tract and reported that an average human secretes 2.5 litres of gastric juice on a daily basis, with an average pH of 2-2.5. In the current study, acidified water at pH 2 was chosen to examine the ability of microencapsulation to protect the *L. acidophilus* for up to 120 min. It is acknowledged that the acidic water used in the study is not equivalent to gastric fluid, since this fluid is more complex in nature and contains acid, enzymes and bile salts. The survival of probiotic bacteria after being subjected to acidic conditions (which can be evaluated by the viable counts) would indicate the potential of encapsulated PB to impart the desired benefits when they arrive at the intestine.

5.2 Materials and Methods

5.2.1 Chemicals and materials

The chemicals and materials used are listed in Table 5.1.

Table 5.1 Chemicals and materials used in this study

Chemicals and Materials	Manufacturer
<i>Lactobacillus acidophilus</i>	Obtained from AUT culture collection
deMan, Rogosa & Sharpe (MRS) broth and agar	Difco Laboratories Inc, Detroit, Michigan
Peptone powder	Bacto™ Peptone, BD, Difco, NSW, Australia
Sodium alginate	GRINSTED® Alginate FD155, Danisco, Australia Pty Ltd., NSW, Australia
Calcium Chloride (CaCl ₂ ·2H ₂ O) and methanol	Ajax Finechem Ltd, Sydney, Australia
Instant skim milk powder	Pam's, New Zealand. Batch no: B2816062, purchased from New World supermarket, Auckland
Concentrated hydrochloric acid and sodium hydroxide	Sigma-Aldrich, St Louis, MO, USA

5.2.2 Microencapsulation of probiotic bacteria with and without apple skin polyphenol extracts

5.2.2.1 Preparation of cell suspension for microencapsulation

The cell suspension of probiotic bacteria, *L. acidophilus*, was prepared according to the procedure described by Shah and Ravula (2000) with some modifications. The inocula for *L. acidophilus* were prepared after thawing the frozen working cultures that had been stored in deMan, Rogosa & Sharpe (MRS) broth with 15% glycerol at -20 °C. An aliquot (10 mL) of sterilised MRS broth in a glass vial was inoculated with 1 mL thawed bacterial culture. The glass vial was then incubated anaerobically at 37 °C in a CO₂ incubator for 18 h. The bacteria were transferred twice successively in MRS broth and incubated at 37 °C anaerobically for 18 h. The bacteria were harvested by centrifugation (Eppendorf centrifuge 5810R, Eppendorf, Hamburg, Germany) at 4 °C at 4000 rpm for 10 min. The supernatant was discarded and the cell precipitate was collected and washed thoroughly with sterilised peptone water 0.1% (w/v). This washing procedure was repeated three times. After discarding the last wash water, the cell

slurry was collected, suspended in peptone water (to obtain approximately 10^{11} cells /mL), and mixed thoroughly using a vortex mixer. The resultant cell suspension was used for microencapsulation.

5.2.2.2 Preparation of encapsulant solution

Sodium alginate solution (1% w/v) was prepared freshly by mixing the required amount of alginate with filtered RO water (i.e., 50% at room temperature and 50% boiling water) using the Silverson L5T high shear mixer (emulsifying screen, 1000 rpm for 2 min, Silverson Machines Inc., East Longmeadow, MA, USA) (Sun-Waterhouse et al., 2011c, Sun-Waterhouse et al., 2011d). The alginate solution was then pasteurised at 85°C for 30 min using the double boiler technique. The resulting mixture was stored in a sterilised and capped Schott bottle at 4°C overnight before being used the following day.

5.2.2.3 Preparation of core solution

Sodium alginate solution (0.5% w/v) was used as the medium for the core solution of the microbeads. Firstly, 2 g of each of the ethanolic or aqueous apple skin PP extracts were separately dissolved in 50 mL RO water (boiled and cooled to room temperature). To this mixture, 50 mL boiling water and 0.5 g sodium alginate were added, followed by homogenising with the Silverson L5T high shear mixer. The alginate-PP solution was then pasteurised at 85 °C for 30 min using the double boiler technique. The resulting mixtures were stored at 4 °C overnight and used the following day. The PB alginate-PP core solution was prepared by adding either 2 mL of bacterial cell suspension (PB-treated core solution) or 0.1% (w/v) peptone water only (control core solution, termed Control bead I), to 10 mL of the above alginate-PP solution. A second control bead (termed Control bead II) was also set up by preparing beads with core solution containing alginate solution and PB in the absence of added PP extracts. The entire process of preparation of final core solution was performed under aseptic conditions under laminar flow workstation (EMAIL AIR HANDLING cabinet, AES environmental Pty Ltd. NSW, Australia). The surface inside the laminar flow hood was wiped with alcohol before and after use and irradiated with UV light for 1 h before using the laminar workstation.

5.2.2.4 Preparation of apple skin polyphenol extracts

The apple skin PP extracts were prepared by either the ethanol or aqueous extraction method as previously described in chapter 2. Hence, all the chemicals and materials used in the PP extract preparation are same as described under section 2.2.1.

5.2.2.5 Microencapsulation of probiotic bacteria and apple skin polyphenol extracts

Co-extrusion encapsulation was conducted using an Inotech Encapsulator (Inotech Encapsulation AG, Dottikon, Switzerland), using the method of Sun-Waterhouse et al. (2011c) and Sun-Waterhouse et al. (2011d). During co-extrusion encapsulation, the nozzle of the encapsulator extruded the core fluid (unfortified or fortified alginate) from a glass syringe and the shell fluid (1% alginate) from a plastic syringe simultaneously into an outer (shell) and inner (core) structure. The core-shell streams dispersed into regular-sized microdroplets under a vibration frequency of 2,723 Hz and were collected and hardened in glass vessels that contained a 200 mL 3% calcium chloride solution. The generated beads were allowed to stand in the CaCl_2 solution for 30 min to allow hardening of the beads. Following this, the beads were collected using a sterile 50- μm nylon mesh, washed with sterile distilled water and transferred to 100 mL Schott Duran glass bottles (wrapped with aluminium foil) containing small amount of 20 mL CaCl_2 solution. The bottles containing beads were then stored at 4°C for another 30 h to allow further hardening of the beads. The beads were then washed twice with sterile distilled water and stored in 20 mL glass vials until required. The entire microencapsulation process was performed aseptically under a Gelman laminar flow hood (Woodlands, Singapore) in dark conditions to avoid interference of light with the PPs. All the glassware used for the process was autoclaved at 121°C for 15 min prior to use.

5.2.3 Microscopic examination of probiotic alginate beads

5.2.3.1 Optical microscopy and Environmental scanning electron microscopy

The bead morphology, size and wall thickness were examined by optical microscopy using a Nikon Eclipse E600 microscope (Nikon Corporation, Chiyoda-ku, Tokyo, Japan) equipped with a Nikon Coolpix 995 3.34 mega pixel camera ($\times 20$, Nikon corporation, Chiyoda-ku, Tokyo, Japan). The bead morphology and size were further examined by environmental scanning electron microscopy (ESEM) using the ESEM mode of a FEI-Quanta-250 scanning electron microscope (FEI, Hillsboro, OR, USA). Before microscopic examinations, the alginate beads were suspended in CaCl_2 solution and an aliquot (20 μl) of this suspension was placed on a Peltier cooled stage (5 $^{\circ}\text{C}$) using carbon tab for adhesion.

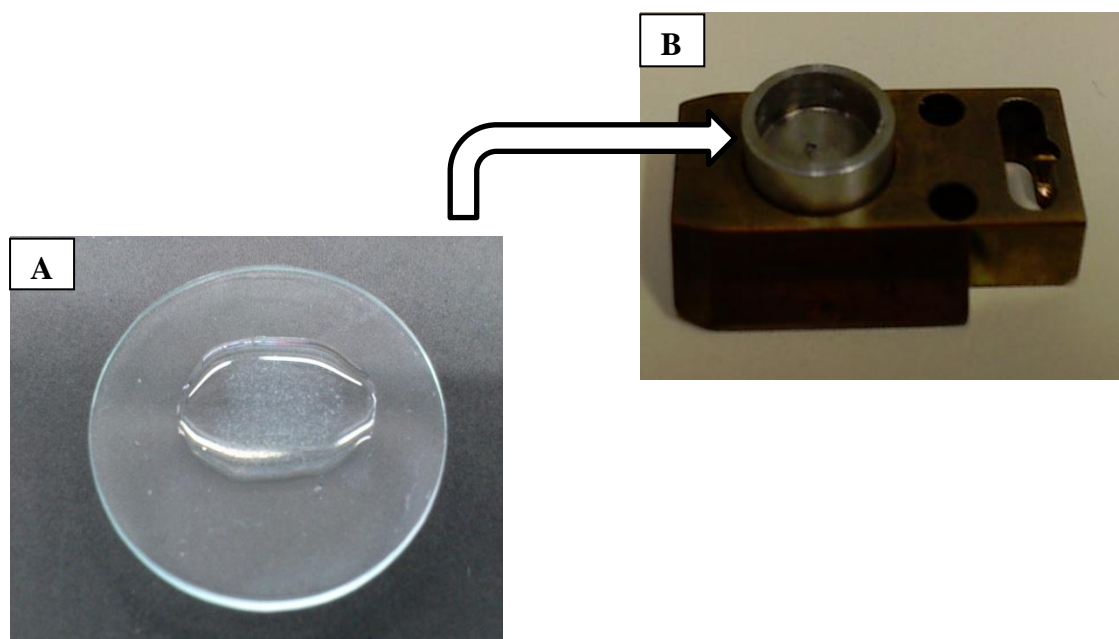


Figure 5.1. Preparation of probiotic alginate beads for ESEM examination: (A) beads suspended in calcium chloride solution and (B) sample holder

The ESEM images of microencapsulated beads were taken at a sample temperature of 5 °C and a relative humidity of 95-100%. The alginate beads containing PB suspended in CaCl_2 solution were collected after most of the liquid was removed with filter papers, and were directly transferred into the sample holder, which was then placed onto a stage in a low-pressure chamber of a FEI-Quanta-250 scanning electron microscope as shown in figure 5.1. To reduce drying during evacuation, 4 drops of distilled water were placed on the stage near the sample. The system was pumped down to give a final humidity of between 95-100% to allow liquid surrounding the beads to slowly evaporate. Viewing and imaging was carried using a gaseous secondary electron detector at a working distance of 5mm with an accelerating voltage of 15 kV and spot size 4. Before and after imaging of individual beads, a low resolution montage image of the region of the image observed was made to record changes due to drying of the sample.

5.2.3.2 Cryo-Scanning electron microscopy

Cryo-SEM examination of alginate beads containing PB was carried out using a Quorum PP2000 cryo-transfer system attached to a FEI-Quanta 250 scanning electron microscope. Prior to commencement of observation, the cryo-stage within the microscope was cooled to lower than -150°C by using a cold nitrogen gas stream and the sample stage in the preparation chamber was cooled to -150°C using liquid nitrogen. A concentrated paste of beads was placed in a 8 mm diameter shallow copper dish placed in the system transfer holder as shown in figure 5.2. The sample was then rapidly frozen in liquid nitrogen slush and transferred under vacuum to the preparation chamber of the system. The raised surface of the frozen paste was fractured using a cooled metal probe and the sample then sublimed at -80°C for 5 min to remove ice from the surface. The sample was then cooled back to -150°C; sputter coated using a gold/palladium target and transferred to the cold stage in the SEM at a temperature of -160°C where it was viewed and imaged at a working distance of 11 mm, with an accelerating voltage of 5kV and spot size 3.

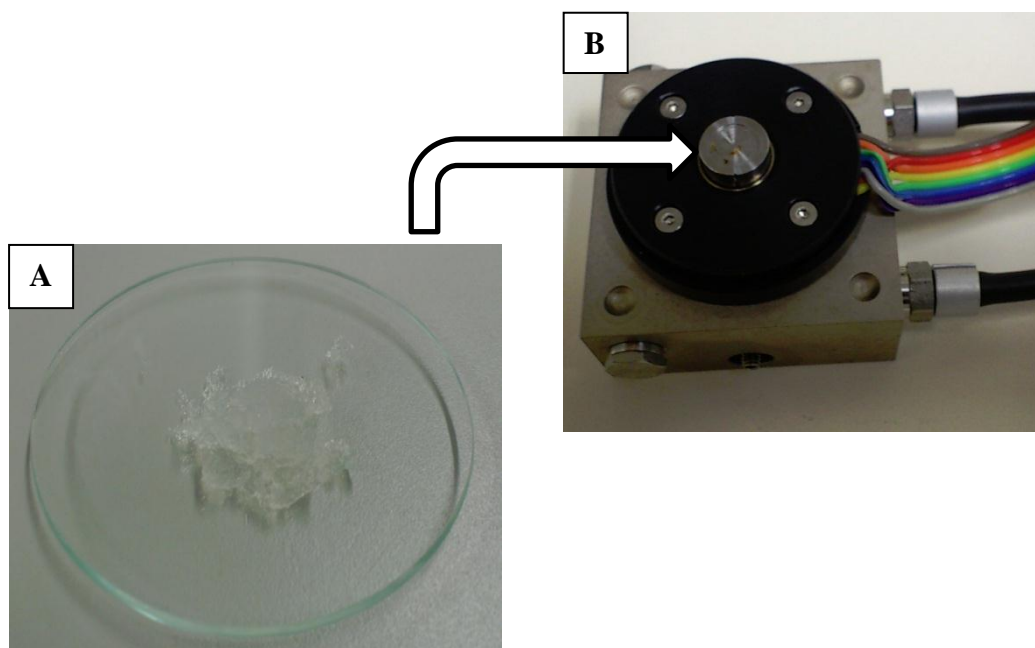


Figure 5.2. Preparation of probiotic alginate beads for Cryo-SEM examination A) concentrated paste of the microbeads and B) sample holder

5.2.4 Enumeration of encapsulated probiotic bacteria and determination of encapsulation efficiency

The enumeration of PB in alginate beads was carried out using the method of Krasaekoopt et al. (2006) with some modifications. Briefly, 1 g of alginate beads were added to 9 mL peptone water (0.1%) and stomached for 120 seconds at normal speed setting using a stomacher (Seward laboratory stomacher® 80, Seward Limited, United Kingdom). This blended mixture was further serially diluted with peptone water and 0.1 mL of appropriate dilution was plated on to MRS agar plates in triplicate, using an automatic spiral plater (Don Whitley Scientific Limited, West Yorkshire, England). The plates were incubated at 37 °C for 48 h anaerobically in a CO₂ incubator (SANYO Electric Co., Ltd, North America, USA). The cell counts were adjusted to a common denomination of per gram solid for comparison. The viable numbers of *L. acidophilus* were expressed as Log CFU/g beads and calculated using the following formula:

$$\text{Viable number} = \text{Log} \frac{\text{Number of CFU}}{\text{Volume plated (mL)} \times \text{total dilution used}}$$

The microencapsulation efficiency (ME) of PB was calculated using a modified equation of Heidebach et al. (2009) as follows:

$$\text{ME (\%)} = \frac{\text{Log CFU/g after microencapsulation}}{\text{Log CFU/g used in core and shell formulation}} \times 100\%$$

To calculate the Log CFU/g of probiotic cells used in the core and shell formulation a known quantity of bacterial cell suspension was mixed with known quantities of core and shell (encapsulant) solution. This was done to account for the dilution of cell suspension in the core and shell solution. This mixture was then serially diluted in peptone water and 0.1 mL of appropriate dilution was plated on to MRS agar plates in triplicate, using an automatic spiral plater (Don Whitley Scientific Limited, West Yorkshire, England). The plates were incubated at 37 °C for 48 h anaerobically in a CO₂ incubator (SANYO Electric Co., Ltd, North America, USA).

5.2.5 Determination of polyphenol content and total antioxidant activity of alginate beads encapsulated with apple skin polyphenol extracts

The alginate beads containing ethanolic or aqueous apple skin PP extracts were freeze dried (Telstar Cryodos-80 Freeze Drier, Telstar Industrial, SL, Terrassa, Spain). The extraction of PPs from the beads were carried out using the method of Sun-Waterhouse et al. (2011d) with some modifications. Freeze-dried encapsulated beads (1 g) were ground in a mortar with pestle for 2 min in the presence of 5 mL methanol until the beads were completely open. The resulting mixture was vortexed followed by centrifugation at 4400 rpm for 3 min, and this step was repeated twice. The supernatant was collected and concentrated in a Labconco CentriVap® Centrifugal Concentrator (Model 78100-01; Labconco CentriVap®, Kansas, MO,

USA) at 40 °C for 3 h under vacuum. The resultant concentrate was freeze dried and then used for TEPC and FRAP analysis as described before (Chapter 3 section 3.2.3).

5.2.6 Preparation and analyses of alginate beads containing probiotic bacteria encapsulated with and without apple skin polyphenol extracts in milk drinks

5.2.6.1 Preparation of milk drinks enhanced with microencapsulated beads containing probiotic bacteria and/or apple skin polyphenol extracts

Skim milk (12% w/v) was prepared as described in Chapter 4 (section 4.2.2.4). The milk was transferred into 250 mL Schott Duran bottles followed by pasteurisation at 85 °C for 30 min using the double boiler technique. 1 g of each of the micro-beads with the following core formulations:

1. Alginate (control beads I)
2. PB + alginate (control beads II)
3. Aqueous PP extract + PB + alginate (Beads III)
4. Aqueous PP extract + alginate (Beads IV)
5. Ethanolic PP extract + PB + alginate (Beads V)
6. Ethanolic PP extract + alginate (Beads VI)

were added to duplicate skim milk samples (cooled to 20 °C). The milk inoculated with probiotic cell suspension (10^{10} CFU/mL) was used as control (termed Milk control with unencapsulated PB). The milk bottles were wrapped with aluminium foil to avoid light penetration and were stored at 4 °C for 50 days. The viability of probiotic bacteria, *L. acidophilus* was evaluated on an interval of 5 days.

5.2.6.2 Enumeration and determination of viability of probiotic bacteria encapsulated in the presence and absence of apple skin polyphenol extracts and then incorporated in milk drinks

The bacterial enumeration for estimating the viability of microencapsulated and co-encapsulated *L. acidophilus* in PP milk drinks was performed by subsampling the following six polyphenol milks drinks:

1. Milk containing microencapsulated aqueous PP extract + PB
2. Milk containing microencapsulated aqueous PP extract
3. Milk containing microencapsulated ethanolic PP extract + PB
4. Milk containing microencapsulated ethanolic PP extract
5. Milk containing microencapsulated PB
6. Milk control containing unencapsulated PB

5 mL milk sample from each of these model milk drinks was withdrawn every 5 days, diluted with 45 mL of 0.1% (w/v) peptone water and blended for 120 seconds at normal speed setting using a stomacher. The subsequent serial dilutions of the blended mixture were prepared in peptone water (in duplicate) and plated onto MRS agar plates (in triplicate) using an automatic spiral plater (Don Whitley Scientific Limited, West Yorkshire, England) in triplicates. The plates were then incubated at 37 °C anaerobically in a CO₂ incubator for 48 h. The viable numbers of *L. acidophilus* were expressed as Log CFU/mL using the following formula:

$$\text{Viable number} = \text{Log} \frac{\text{Number of CFU}}{\text{Volume plated (mL)} \times \text{total dilution used}}$$

5.2.6.3 Survival of microencapsulated and unencapsulated probiotic cells at pH 2

The estimation of survival of free unencapsulated and microencapsulated *L. acidophilus* cells under acidic conditions was performed following the method of Nag (2011) with some modifications. The acidic solution was prepared by addition of concentrated hydrochloric acid to sterilised Milli-Q water till its pH dropped to 2. One gram of the following beads : Control beads I, Control beads II, Beads III and Beads VI were added to glass vials containing 9 mL of the pre-warmed (37 °C) acidified water (pH 2) for 120 min (in duplicate). 1 g of cell suspension of unencapsulated PB was also added to 9 mL acidic solution as the control (termed “unencapsulated *L. acidophilus*”). On an interval of 30 min, aliquots of subsample were taken out from each of the combinations. The pH of the medium was raised to 7.0 immediately with 0.1N NaOH to destabilize the gelled matrix for easy breakdown of beads. The pH meter electrode was sterilised with chlorine solution and rinsed with sterilised Milli-Q water. The release of the live bacterial cells was facilitated by stomaching the neutralised suspension and plating the appropriate dilutions onto MRS agar. The plates were then incubated at 37 °C for 48 h in a CO₂ incubator. A negative control in peptone water (pH 7) at time zero was set up to measure the initial cell population. The cell counts were adjusted to a common denomination based on unit gram solid for comparison. The microsphere morphology of the incubated beads was evaluated by optical microscopy.

5.2.7 Statistical analysis

Experimental data were statistically analysed using Minitab® (version 16) software (Minitab Inc., State College, Pennsylvania, USA) with Analysis of Variance (ANOVA), using a repeated measures design for viability and adhesion assays. At least two observations per analysis were performed and the results were expressed as mean values and standard deviation of the mean. The difference is considered to be of significant importance at more than 95% confidence interval ($P < 0.05$). When significant differences occurred, Tukey's HSD (Honestly significant difference) test was employed to examine where that difference occurred.

5.3 Results and Discussion

5.3.1 Microscopic examination of microencapsulated beads

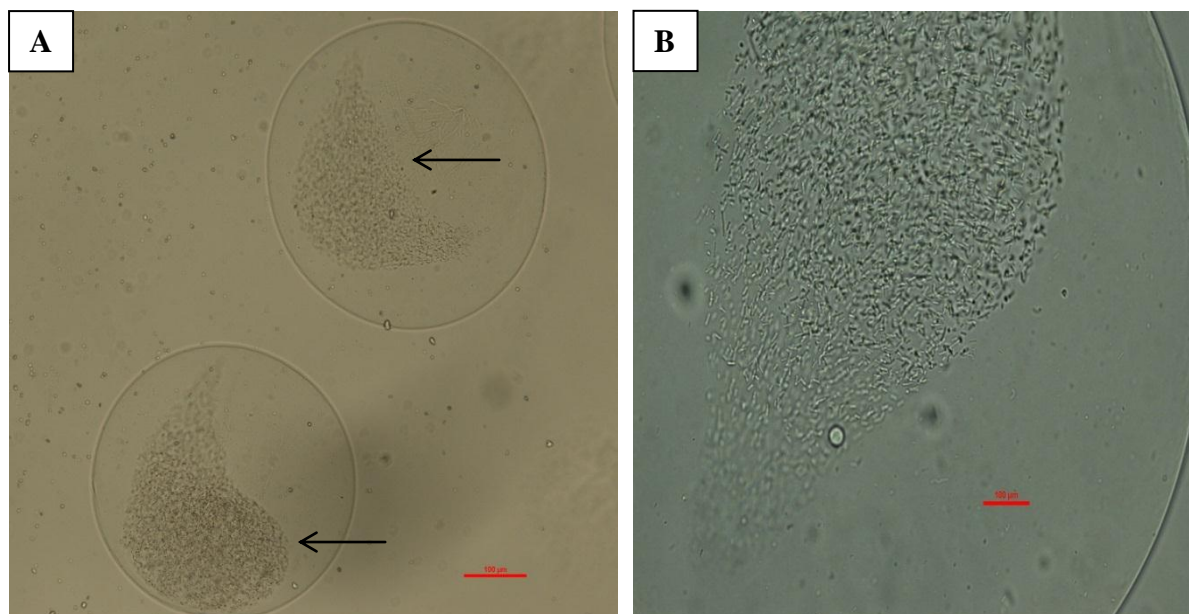


Figure 5.3. Alginate beads containing encapsulated *L. acidophilus* cells (as pointed with black arrows) examined by optical microscope at (A) 10× magnification, (B) 40× magnification

Optical micrographs (Figures 5.3 A and B) revealed that the alginate beads carrying PB were successfully encapsulated in spherically shaped beads. The beads mostly had smooth surfaces with a constant size. The encapsulated bacteria can be visualised by optical microscope to be located inside the alginate wall, and the interface between the core (PB-alginate solution) and alginate-shell appeared to be in undefined pattern. The diameter of the whole beads ranged from 100 – 300 µm. Previous studies reported the smooth surface of the beads prepared using shell alginate at a higher concentration (usually 3%) (Chen et al., 2005, Sheu and Marshall, 1993, Sun-Waterhouse et al., 2011c). The beads of the current study, which were prepared, using 1% alginate, appeared to be strong, with minimal wrinkles and no breakage was observed (see figure 5.4).

The ESEM examinations also revealed the smooth surface and roughly spherical shape of the alginate beads of this study (Figure 5.4). The diameter of the encapsulated beads was 100 µm, which is in agreement with the estimation based on optical images. ESEM micrographs show dented surfaces of the encapsulated beads (Figure 5.4), which could have

resulted from the water drying out of the beads in the chamber during ESEM examination. This is also evident from the images taken at the beginning and end of the ESEM examination (Figures 5.5 A and B, the time difference was 1 h).

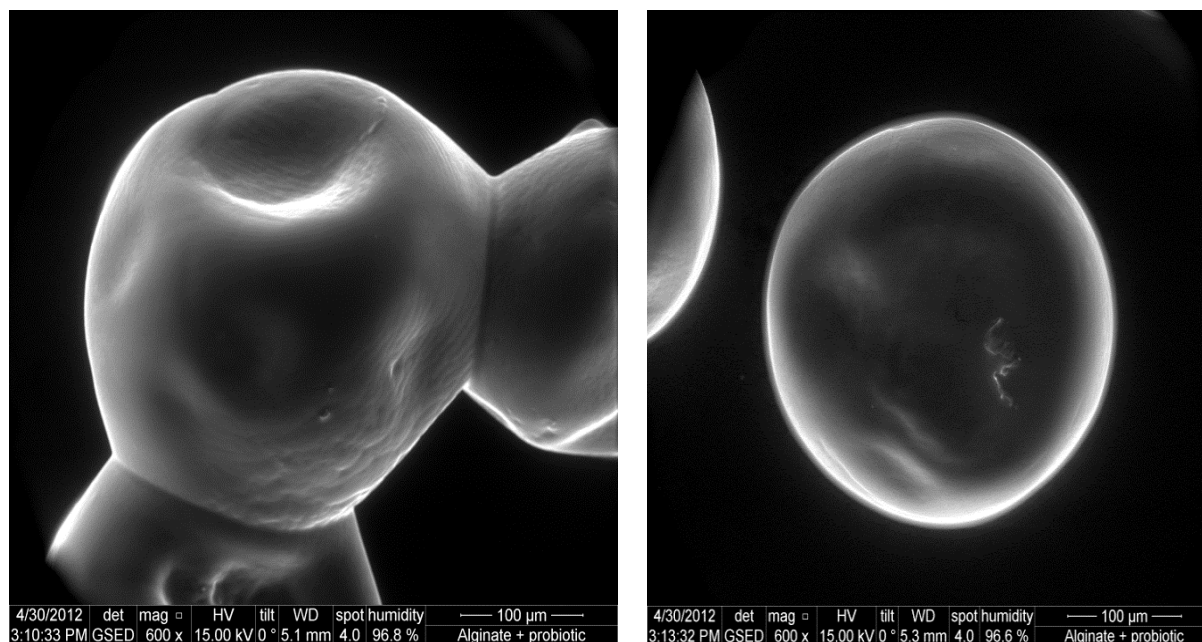


Figure 5.4. Alginate beads containing PB examined by ESEM at 600 × magnification

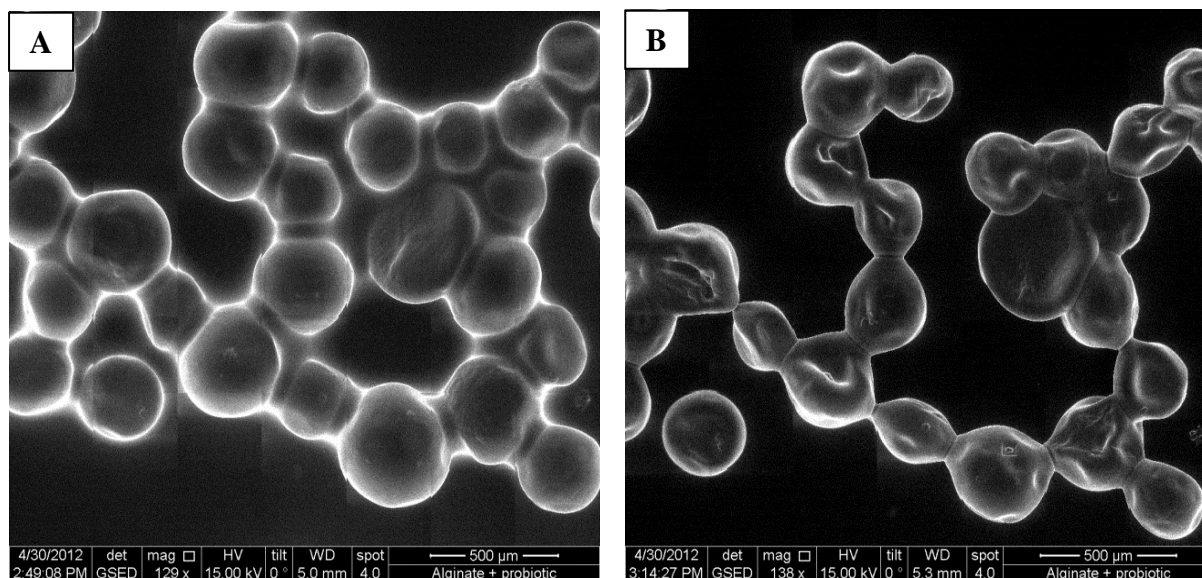


Figure 5.5. Montage ESEM images of the probiotic alginate beads taken at the beginning (A) and at the end (B) of microscopic examination of the beads

The size and appearance of alginate beads were found to be influenced by numerous factors, including the type and concentration of alginate, as well as variables related to the encapsulator, such as the setting of shell- and core-syringes and the diameter of the extruder orifice (needle) (Mortazavian et al., 2007). No *L. acidophilus* cells were detected on the external surface of the beads indicating that the encapsulation was complete and probiotic cells were not exposed externally.

The cryo-SEM images of intact beads are displayed in figure 5.6. The probiotic alginate beads appeared to be slightly spherical to oval shaped after cryofixation (high pressure freezing) by liquid nitrogen slush. Quick removal of water from the beads via sublimation was thought to be responsible for the bead shrinkage and the ice crystals on the bead surface. The alginate beads remained intact without any detectable breakage in the beads. Figures 5.7 and 5.8 show the fractured bead and its internal structure and associated matrix components respectively. Figure 5.7 shows an alginate bead in oval shape with an internal laminar-like network structure that contains “voids” or “channels”. Figure 5.8 is a magnified image of figure 5.7, showing a large number of rod shaped *L. acidophilus* cells (as pointed by white arrows) on the internal laminar-like structure. The laminar-like structures inside the beads were possibly formed by the frozen alginate polymers along with ice crystals due to freeze fracturing.

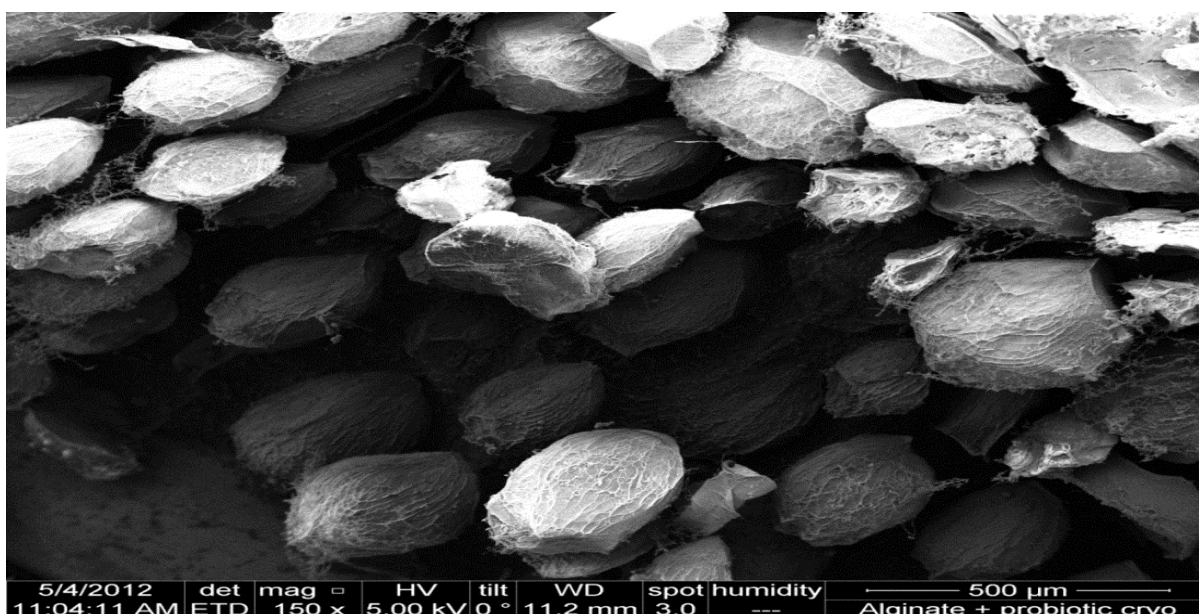


Figure 5.6. Cryo-SEM image of intact alginate beads containing probiotic cells at 150 × magnification

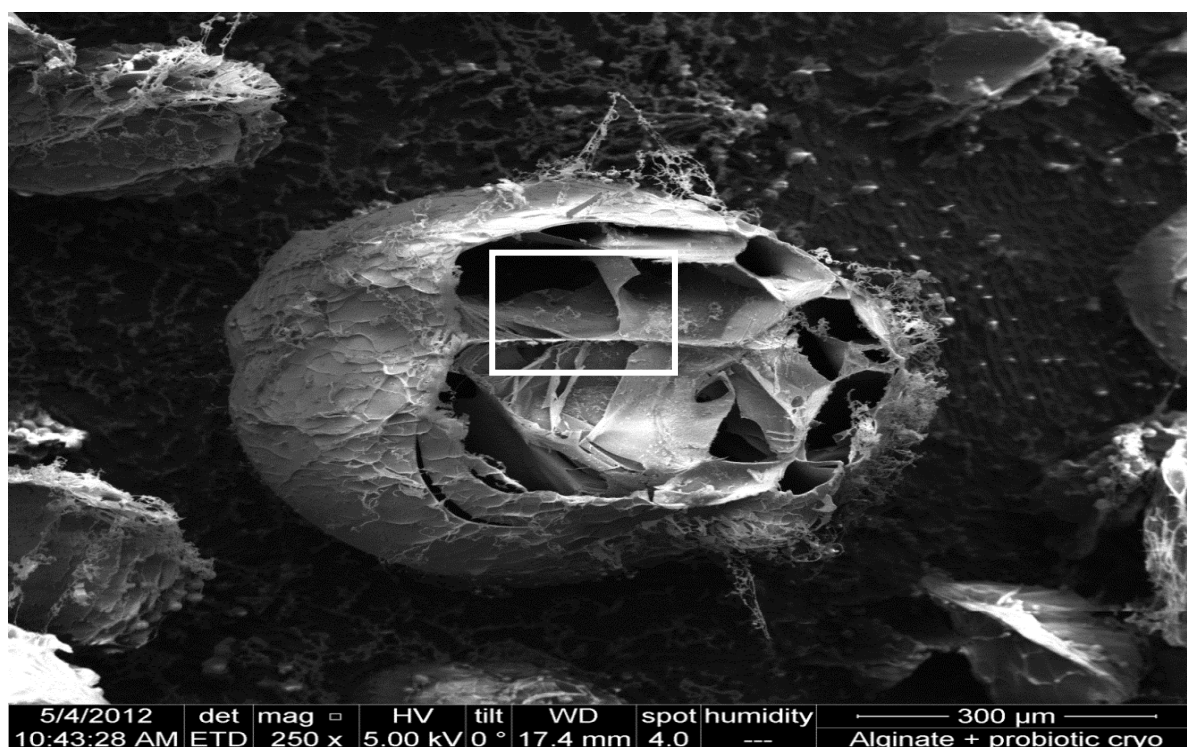


Figure 5.7. Cryo-SEM image of fractured alginate bead containing probiotic *L. acidophilus* cells (Square area will be magnified in figure 5.8)

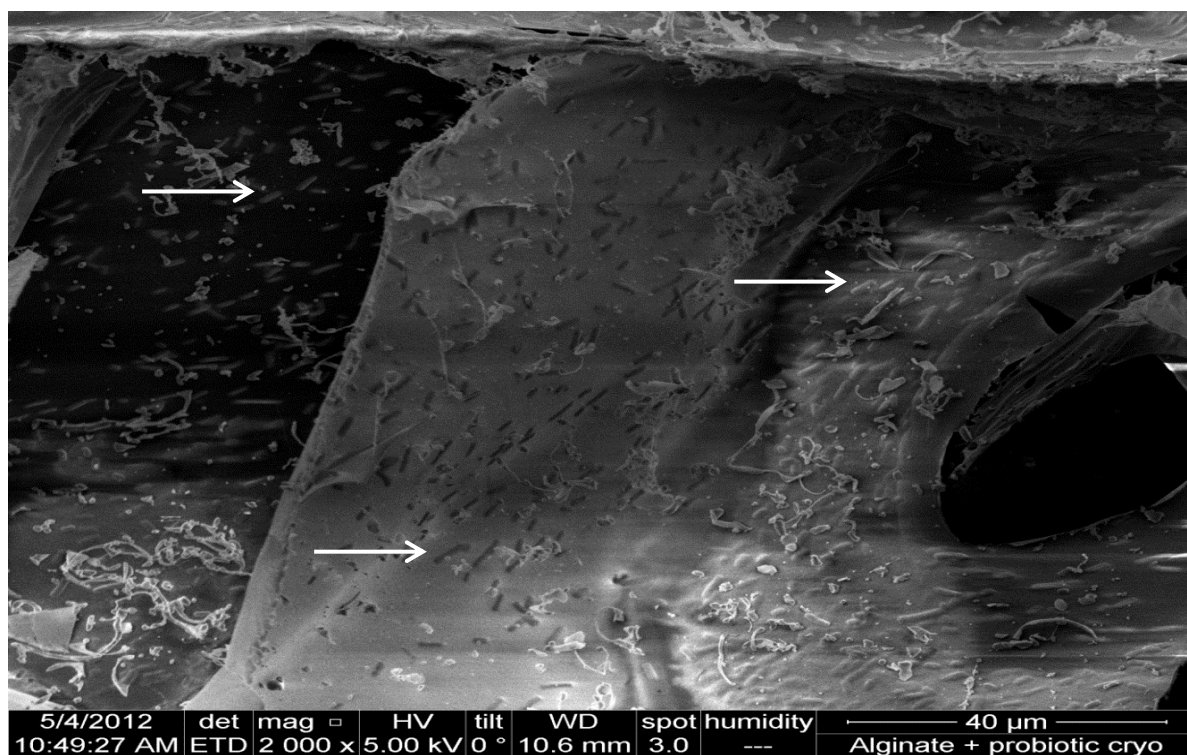


Figure 5.8. Magnified cryo-SEM image of Figure 5.7, showing the internal structure and contents of the encapsulated bead (probiotic cells pointed with white arrows)

5.3.2 Microencapsulation efficiency

The microencapsulation efficiency (ME) of alginate beads containing PB and a PP extract (either ethanolic or aqueous) along with PB was evaluated and is listed in table 5.2. The overall ME of microencapsulated beads was slightly higher than 96%. Thus, 0.5% and 1% alginate solutions were an effective core medium for carrying PB and efficient shell material respectively to generate intact and smooth beads (evident by optical and SEM images). The high ME can be attributed to the selection of a suitable alginate and feasible concentration, as well as the gentle and rapid operations involved in the whole co-extrusion microencapsulation process, which led to minimal cell injury and efficient protection of PB as suggested by Mortazavian et al. (2007).

Table 5.2. Microencapsulation efficiency of alginate beads containing apple skin polyphenol extracts

Microcapsule type	Log CFU/g beads	ME (%)
Ethanolic PP extract + PB	10.04 ± 0.02	96.69
Aqueous PP extract + PB	10.02 ± 0.03	96.35
PB (control)	10.00 ± 0.02	96.46

Most of the published studies have used an oil-based emulsion technique to encapsulate PB (Annan et al., 2007, Capela et al., 2006, Ding and Shah, 2007, Ding and Shah, 2009, Homayouni et al., 2008, Mandal et al., 2005). The use of oil imparts sensory effects to the food which limits the application of the technique, particularly where no oil is desired in probiotic foods such as milk drinks and juice. The co-extrusion technique has been successfully used by Ying et al. (2007) for encapsulating *L. rhamnosus*, using kiwifruit mucilage in alginate beads in which the design of the core and shell solutions are quite different from this current study. Furthermore, the use of a high concentration of initial cell suspension may also contribute to the high ME of the alginate beads in this study.

5.3.3 Polyphenolic content and total antioxidant activity of alginate beads containing encapsulated apple skin polyphenols

The TEPC content of the alginate beads containing encapsulated ethanolic PP extract appeared to be slightly lower than that with encapsulated aqueous PP extract, but this was shown to be insignificant ($P>0.05$) after statistical analysis (Table 5.3). The TAA of the alginate beads with encapsulated aqueous PP extract was statistically significantly higher than that with encapsulated ethanolic PP extract. These results show the feasibility of using aqueous alginate solution for co-extrusion encapsulation, which presents opportunities of using these encapsulated beads to carry significant amounts of PPs in foods.

Table 5.3. Total extractable polyphenol content and total antioxidant activity of beads containing encapsulated apple skin PP extracts

Alginate Beads	TEPC (mg catechin eq./g beads)	TAA (mg Trolox eq./g beads)
Ethanolic PP extract	2.61 ± 0.12^a	3.05 ± 0.05^a
Aqueous PP extract	2.73 ± 0.06^a	3.44 ± 0.04^b

Data are expressed as mean \pm standard deviation. Different lowercase superscript letters (within the same column) indicate statistically significant differences at $P<0.05$

5.3.4 Viability of probiotics co-encapsulated with apple skin polyphenol extracts

The effects of apple polyphenol extracts on the viability of unencapsulated, microencapsulated and co-encapsulated (with aqueous and ethanolic PP extracts) *Lactobacillus acidophilus* in model milk drinks during a storage period of 50 days are shown in figure 5.9. Compared with the unencapsulated (free) PB in milk, the cell death of PB was much lower when PB were encapsulated alone or together with an apple skin extract in alginate beads in the milk over the entire storage period. Decrease of only 0.13-Log and 0.16-Log in viable cells were detected for the *L. acidophilus* co-encapsulated with aqueous or ethanolic PP extract, respectively, after 50 days. The decrease in cell count was significantly higher ($P<0.05$) for the

unencapsulated PB (1.1-Log) and the *L. acidophilus* encapsulated alone (0.34-Log) respectively. Also, aqueous apple skin extract was found to be equally effective ($P>0.05$) in protecting *L. acidophilus* cells in milk as the ethanolic extract. The PB co-encapsulated with the ethanolic extract had significantly the same viability as that with PB microencapsulated alone (in the absence of PPs).

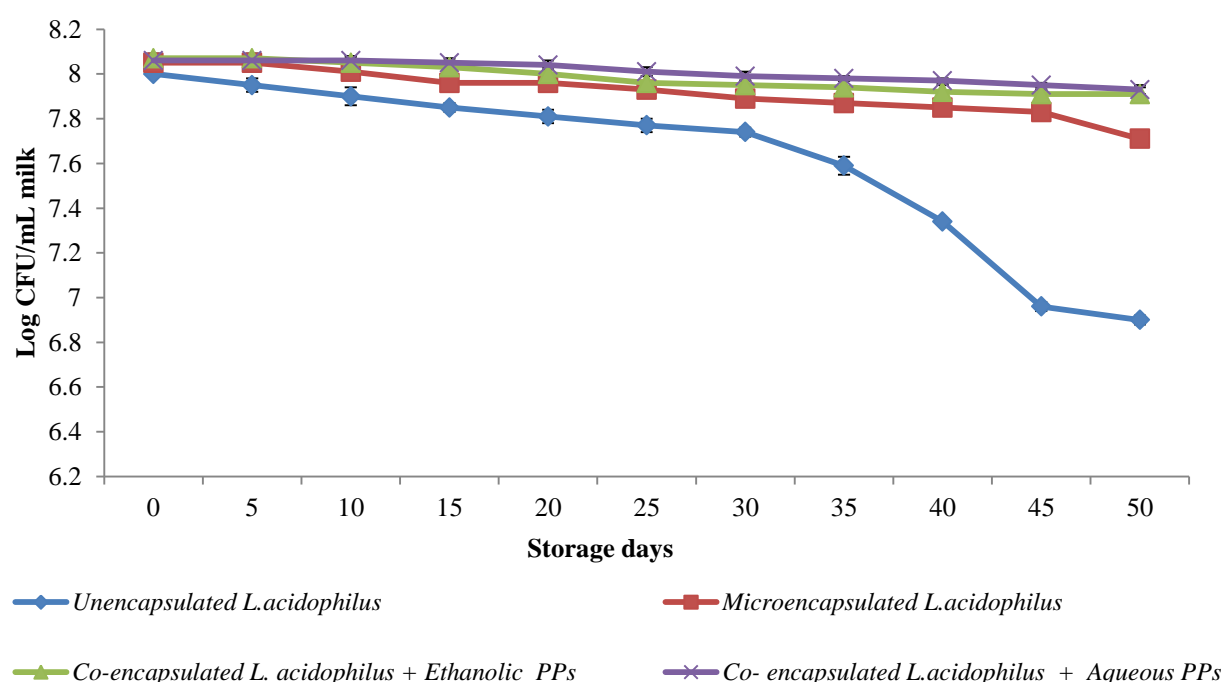


Figure 5.9. Influence of apple polyphenol extracts on the viability of co-encapsulated *Lactobacillus acidophilus* in refrigerated (4 °C) model milk drinks

In the case of viability of unencapsulated *L. acidophilus* cells in the refrigerated milk drinks, the cell number declined from 8 Log CFU/mL on Day 0 to 6.9 Log CFU/mL on Day 50. The greatly reduced cell counts of unencapsulated *L. acidophilus* cells under refrigerated storage in milk drinks was reported in chapter 4 (section 4.3.1). Even though in this case, the number of viable probiotic cells in the milk drinks after 50 days did not fall below the minimum requirement of 10^6 CFU/mL, it is still significantly ($P<0.05$) lower than the PB co-encapsulated with PP extracts. Microencapsulation was found to prolong the viability of PB over 50 days, especially when encapsulation was carried out in the presence of the aqueous PP or ethanolic PP extracts.

The exact mechanism of action of PPs underlying the preservation of the viability of *L. acidophilus* in the alginate beads needs to be elucidated; the antioxidant activity of the apple skin extracts is one of the possible reasons. As pointed out in earlier chapters, the chemical structure and concentration of the individual PPs in these extracts are responsible for the difference between the aqueous and ethanolic extract in influencing proliferation of probiotic bacteria (Duda-Chodak et al., 2008). PPs were found previously to bind to the bacterial surface and significantly enhance the total oxidant-scavenging capacities of bacteria (Koren et al., 2009). Oxidative stress is one of the possible factors that negatively affect the viability of PB, particularly catalase- and peroxidase-negative LAB in the food matrices (Rochat et al., 2006). Polyphenols on their own are well known antioxidants and antimicrobial agents (Sun-Waterhouse, 2011) that can reduce the oxidative stress in food systems and prevent the growth of pathogenic and food-spoilage microorganisms. Moreover, polyphenols have been demonstrated to improve the growth and attachment of probiotic bacteria (Parkar et al., 2008). Hence, the co-encapsulation of PB with PPs may offer a means of maintaining the viability of PB, in addition to providing several health benefits, including an anti-neurodegenerative effect, anti-ulcer activity, anti-carcinogenic effect, anti-mutagenic activity and anti-inflammatory activity (Sun-Waterhouse, 2011).

The selection of core material ingredients for encapsulation is vital. Many studies have confirmed the efficacy of various prebiotics such as Hi-maze, fructooligosaccharides (Raftilose), inulin (Raftiline) and isomaltooligosaccharides in improving the viability of probiotic bacteria using the co-encapsulation technique (Capela et al., 2006, Chen et al., 2005, Iyer and Kailasapathy, 2005). The presence of uronic acid, a derivative of pectin, in the apple skin extract could also have contributed to the protective effect on the PB in the milk. Support for this conclusion comes from the use of the cryoprotectant UnipectineTM RS 150, which has been shown to enhance the viability of *L. casei* by 7% during the 4 weeks of refrigerated storage in yoghurt (Capela et al., 2006). Microencapsulation using alginate was reported to enhance the viability of PB in freeze-dried yoghurts in their study. Co-encapsulation of *L. acidophilus* strains with Hi-maize was reported to improve the viability in synbiotic yoghurt (Iyer and Kailasapathy, 2005). Ying et al. (2007) utilised kiwifruit mucilage to encapsulate *L. rhamnosus* though such encapsulated bacterial cells did not survive the acidic environment. No

previous studies have reported the influence of PPs on the viability of PB using microencapsulation. The current work is a pilot study in this regard.

5.3.5 Survival of probiotic bacteria encapsulated in the absence or presence of apple skin extract under acidic condition

The survival of probiotic microorganisms and maintaining sufficient PB numbers (at least 10^6 CFU/mL) during the passage along the human gastro-intestinal tract is desired in order to impart the health benefits. The pH to which PB are exposed can be as low as 2. Thus, successful delivery of probiotic cells to the intestinal area remains a challenge (Annan et al., 2007). Microencapsulation of PB has been suggested to be an approach to provide such protection (Ding and Shah, 2007). In the current study, probiotic *L. acidophilus* encapsulated alone or co-encapsulated with either aqueous or ethanol apple skin PP extract, was subjected to acidified water at pH 2 for 120 min (a simplified acidic model system for preliminary survival study). The survival of microencapsulated and unencapsulated (free) cells of *L. acidophilus* was both evaluated.

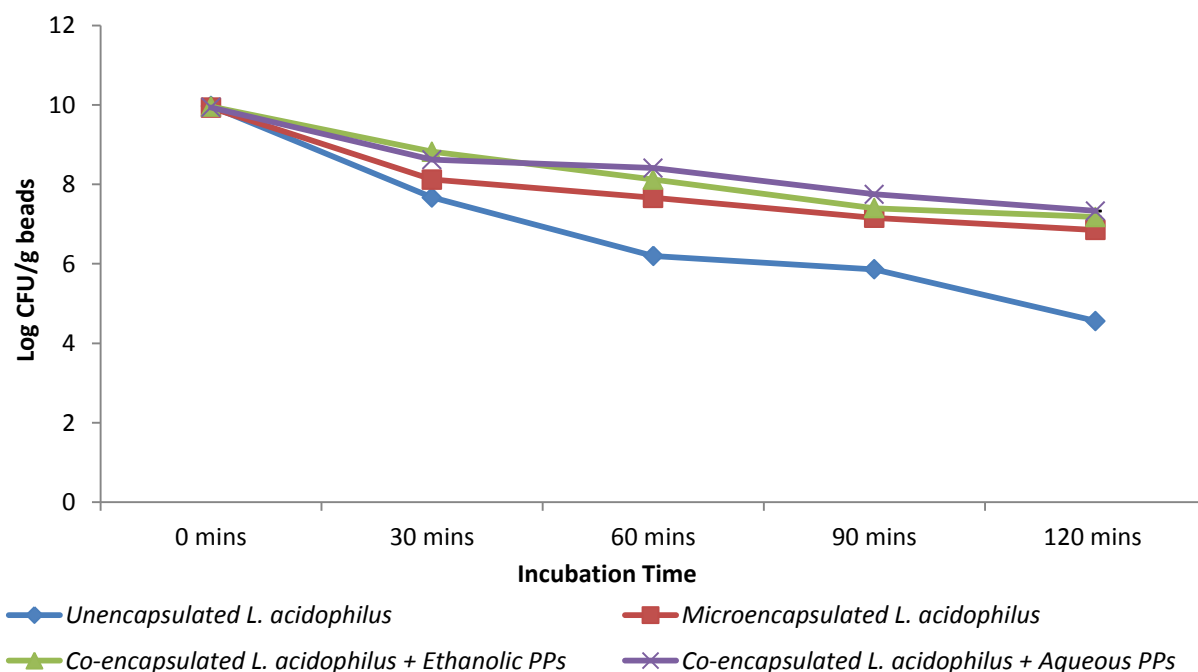


Figure 5.10. Survival of co-encapsulated, encapsulated and unencapsulated cells of *L. acidophilus* during incubation in acidic condition (pH 2)

Encapsulation with alginate beads protected PB against the acidic condition (at pH 2), and such a protection was greatly enhanced when encapsulation was performed in the presence of an apple skin PP extract (Figure 5.10). Cell loss of 5.41-Log was noted for unencapsulated *L. acidophilus* cells after being exposed to acid environment, at the end of 120 min. This substantiates with the study by Heidebach et al. (2010) where 5 Log reduction of *L. paracasei* after 90 min of incubation at pH 2.5 was reported. The viability of free unencapsulated cells decreased greatly with the time of exposure to acidic condition. A much lower degree (3.08-Log) of loss in cell count of probiotic cells ($P<0.05$) was obtained when the PB was encapsulated with alginate polymer. Statistical analysis revealed that significant ($P<0.05$) protection against acidic condition was found between the *L. acidophilus* co-encapsulated with the aqueous apple skin PP extract (cell loss of 2.61-Log) and ethanolic skin extract (cell loss of 2.78-Log) compared with that encapsulated alone. The results regarding the viability of microencapsulated cells in this study are in agreement with a previous study by Chandramouli et al. (2004) in which a substantial protection in viable *L. acidophilus* at pH 2 was found when the cells were microencapsulated with alginate.

The integrity of the wall material of the alginate bead after exposure to acidified water at pH 2 for 120 minutes is shown by the optical microscopic images (Figure 5.11). The alginate beads retained their integrity at pH 2 after 2 h. No breakage of beads or any damage to the alginate beads was noted during microscopic examination. The spherical shape of the beads was retained after the exposure to the acidified water at pH 2 for 2 h (Figure 5.11 A “before exposure” and 5.11 B “after exposure”). Only slight reduction in the bead size was observed. This suggests that the alginate beads prepared in this study have provided a strong and effective protective barrier to PB against the harsh environmental conditions. In a previous study, alginate beads generated using extrusion technology also provided good protection to PB against simulated gastric juice (pH 1.5) (Muthukumarasamy et al., 2006).

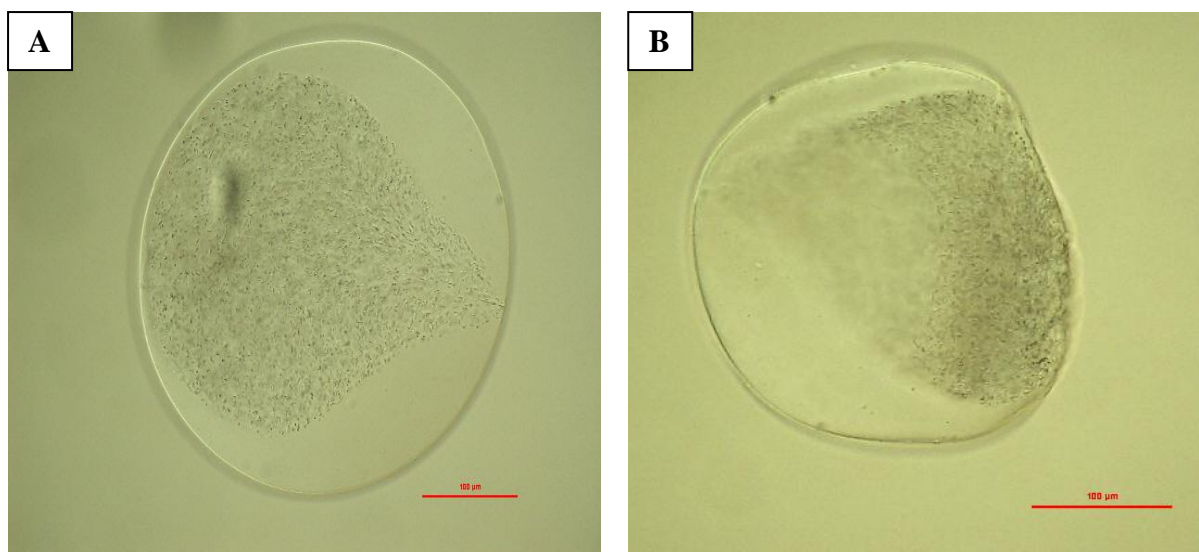


Figure 5.11. Optical microscopic images of probiotic alginate beads incubated at pH 2 at the end of 120 min of acidic incubation (magnification 20 \times). (A) Before acidic exposure, (B) After acidic exposure). The scale bar indicates 100 μm

5.4 Conclusions

The microencapsulation and co-encapsulation of PB with apple skin PP extracts was found to provide enhanced protection to the survival of probiotic *L. acidophilus* in milk drinks stored for 50 days under refrigeration (4 °C). This indicates that the microencapsulation offers the opportunity to deliver live probiotics at the minimum benefiting dose through a food matrix like milk. The cell loss of PB in the milk was much lower when they were either co-encapsulated together with the apple skin extracts, or encapsulated alone, compared with the unencapsulated *L. acidophilus* cells over the entire storage period. The alginate beads encapsulated with the ethanolic or aqueous apple skin extract provided much greater protection for the survival PB cells in the milk, suggesting the efficacy of apple skin extracts in maintaining the viability of PB. This protective effect could possibly be attributed to the antioxidant activity of PPs in the skin extracts, which are known to reduce the oxidative stress in food systems (Sun-Waterhouse, 2011).

The alginate polymer used as encapsulant material proved to be effective in maintaining the integrity of the final beads generated in this study, as revealed by the microscopic examinations. The alginate beads containing PB were noted to have spherical shape, smooth surface and constant size. The beads prepared using 1% alginate shell solutions were strong and had minimal wrinkles, with no breakage. Remarkably high microencapsulation efficiency (ME) was achieved (>96%), in the absence or presence of apple skin extract, with alginate polymer. This may be attributed to the non-toxic nature of alginate, as well as to gentle operations during the co-extrusion microencapsulation process. This further suggests that alginate at the concentrations used in this study is an effective encapsulation and core medium to carry PB.

The high TEPC and TAA values of the beads indicate the excellent efficacy of the co-encapsulation technique used in the study to incorporate most of the skin extracts used in the core solution. This in turn, suggests that the co-encapsulation of PB with apple skin PP extracts can be used to deliver the bio-functionalities of both these beneficial components in their respective active forms. This was further confirmed from the maintenance of viability of microencapsulated probiotic cells above the required minimum level throughout the storage trial. The microencapsulation and co-encapsulation significantly prolonged the viability of

probiotic *L. acidophilus* in the milk drinks compared with that of free unencapsulated cells. Hence, it can be concluded that the microencapsulation technique can be employed to deliver live probiotic cells through foods in order to get the desired benefits from these beneficial microorganisms.

Furthermore, microencapsulated and co-encapsulated *L. acidophilus* probiotic cells demonstrated excellent resilience in the acid test for 120 min in comparison with the unencapsulated cells. The beads were seen to maintain their spherical shape without any damage at the end of incubation in acidic water at pH 2 for 120 min. This suggests that the alginate beads carrying probiotic and apple skin PP extracts can provide protection to PB in the acid conditions encountered in the upper digestive tract. The survival of PB in the hostile condition of the GI tract is important for them to be able to impart the desired health benefits to the host. The probiotic bacteria should be metabolically stable and active in the product and survive the transit through the hostile upper digestive tract in large numbers for them to confer beneficial effects when in the intestine of the host (Gilliland, 1989). Hence, microencapsulation serves to co-entrap antioxidant PPs that have the ability to selectively stimulate the activity and/or proliferation of probiotic and beneficial intestinal bacteria, offering the possibility of using microencapsulation to deliver multiple bioactive ingredients.

Chapter 6

Conclusions

6.1 Summary of main findings

The main purpose of this study was to investigate the beneficial interactions between probiotics and polyphenols (PPs), which promote the viability and adhesion of probiotic bacteria. The ultimate goal of the investigation was to generate useful information to guide the development of a functional dairy beverage carrying significant quantities of probiotics and antioxidant PPs. Interactions (via an inhibitory, neutral or stimulatory effect) were previously found possible between colonic microflora and PPs (Parkar et al., 2008). Various types of fruit PPs demonstrated different effects (stimulatory or inhibitory) on different strains of yoghurt starter cultures in the study by Sun-Waterhouse et al. (2011e). This current study stemmed from these previous findings, but considered probiotic bacteria (PB), represented by *L. acidophilus*, and a cost-effective PP source, apple skin.

This current study has commercial value-added and industrial relevance. This study aimed to present a solution to the technical issues related to low viability and subsequent insufficient attachment of PB to the gut lining, which are commonly encountered problems during the manufacturing and consumption of probiotic-containing products. The use of apple skin as a source of PPs in this study also provides an approach for utilising apple processing by-product wastes. Little was previously reported on the use of apple skin extract in relation to PB activities. Two types of apple skin extracts were used in this study, with one being produced using the conventional ethanolic extraction method and the other using a cost-effective aqueous method. Microencapsulation was also employed in this study to preserve the bioactive components like PB and PPs in milk drinks, by which maximum PB and PP quantities were retained in the final milk drink product.

The first part of this project (Chapter 3) focused on the comparison of the two polyphenol extracts from apple skin, in terms of the efficiency of the extraction method, as well as their total polyphenol content, antioxidant activity, pectin fibre and vitamin C contents. Results demonstrated that the two polyphenol extracts from apple skin prepared using either

ethanol or acidified water, had significantly different total extractable polyphenol content (TEPC) and total antioxidant activity (TAA). HPLC analysis showed that the aqueous apple skin PP extract contained higher concentrations of the majority of individual species of PPs than that in the ethanolic skin PP extract. The examinations of the TEPC and TAA of apple skin extracts not only indicate their potential as functional ingredients, but also measure their ability to influence PB.

The aqueous extract preparation method using acidified water (pH 3) was shown to be a more efficient extraction for PPs in apple skin than the ethanolic extraction method. Similar findings were previously reported but for other research purposes (Strack, 1997, Sun-Waterhouse et al., 2011b, Sun-Waterhouse et al., 2008b, Williamson et al., 2000). The dietary fibre pectin and vitamin C contents of the aqueous and ethanolic extracts were similar. The differences in the proportions of individual PP compounds and the TAA between the aqueous and ethanolic extracts may largely be responsible for their different impact on probiotic activities. Moreover, the aqueous extraction method has additional advantages over the ethanolic method in terms of extraction efficiency, availability of resources and cost-effectiveness (Sun-Waterhouse et al., 2011b, Sun-Waterhouse et al., 2008b). These findings have provided the justification for using these apple skin extracts for wider food applications.

The second part of the study (Chapter 4), examined the influence of ethanolic and aqueous apple skin PP extracts on the adhesion and viability of PB. To elucidate the effect of individual PP compounds on the viability of PB, both the crude extracts from apple skin and purified PP chemicals were added to milk drinks containing *L. acidophilus* as PB. The aim of this part of study was to evaluate the effects of apple PPs (crude and purified extracts) on the viability of PB in the milk drinks during refrigerated storage at 4 °C over 50 days. The two apple skin extracts, compared with the purified PPs, were highly efficient in maintaining the viability of *L. acidophilus* in the milk over 50 days at either concentration of 1% and 2%. Among the purified PP compounds used, rutin showed the highest efficacy in maintaining probiotic viability, whereas, *p*-coumaric acid and quercetin were the least effective. It is satisfactory to note that the two apple skin PP extracts and the six purified PP compounds were all able to maintain the viability of PB in the PP-enhanced milk above the lowest limit of the required PB level of 10^6 CFU/mL (when the initial probiotic cell concentration was approximately 10^7 CFU/mL). The steady loss of *L. acidophilus* viability in the control milk

(carrying only the PB without added PPs) to below the required minimum PB level was observed after 50-day's storage, suggesting the inability of *L. acidophilus* cells to survive in milk under refrigeration in the absence of PPs.

Moreover, in the current study, chlorogenic acid and phlorizin were not found to cause inhibition of *L. acidophilus* at the concentrations used in the current study, in contrast with results reported by Sun-Waterhouse et al. (2011e). The difference in the types of PPs and concentrations between this current study and the previous work may account for such discrepancies. The finding that glycosylated forms of purified PP compounds were more effective in preserving the viability of probiotics than their aglycone counterparts, are in good agreement with those of Sun-Waterhouse et al. (2011e) who concluded that the effect of PPs vary, depending on concentration, structure and probiotic strain.

The apple skin PP extracts and the purified PPs were also proven to be effective in enhancing the adhesion of PB to the hydrophilic tissue culture treated plate surface. The plate surface in this current study is a surrogate for the surface of intestinal cells where PB attaches. It is acknowledged that the mucosal lining of the intestine is much more complicated than the abiotic polystyrene. Despite the limitation of this current adhesion assay, the crystal violet attachment assay is still a near-native attachment assay providing useful indications of the positive effect of PPs on the adhesion properties of *L. acidophilus*. The efficacy of aqueous apple skin extract was shown to be higher in promoting the adhesion of PB under both aerobic and anaerobic conditions and at both incubation temperatures (37 and 4 °C) in this study. The ethanolic apple skin extract and rutin were found to be the second best PP sources to promote the adhesion of PB, followed by epicatechin and phlorizin under either incubation conditions and at either temperature. These results suggest that apple PPs could have the ability to promote adhesion of PB along the intestine (at body temperature) and in the oral cavity (at a temperature approaching that of chilled drinks). Quercetin was seen to be least effective amongst the purified PP compounds tested in terms of aiding the adhesion of *L. acidophilus*. The anaerobic incubation favoured more adhesion than aerobic condition when *L. acidophilus* cells were incubated in the medium with or without added PPs.

The precise mechanism by which these PPs enhance the adhesion and viability of *L. acidophilus* remains unknown. However, the positive roles of purified PPs in promoting

probiotic viability in this study suggest similar effects from the PPs present in the apple skin extracts (shown in the HPLC profiles), and the substantial TAA of the apple skin extracts may be responsible for their positive action towards PB, as suggested by previous studies by Duda-Chodak et al. (2008), Koren et al. (2009) and Parkar et al. (2008). The results obtained from the viability and adhesion assays of this current study indicate that apple skin can be used as a potential antioxidant ingredient to influence the viability and adhesion of PB.

The third part of the research (Chapter 5) employed the co-extrusion technology to microencapsulate PB and PPs together to preserve these bioactives in the final milk drinks. The efficacy of co-encapsulation of PB and apple skin PP extract on the viability of PB in the refrigerated milk drinks and their survival in acidic condition was also evaluated. Alginate polymer has demonstrated its effectiveness in encapsulating *L. acidophilus* cells, as evident from the optical and scanning electron microscopy. The alginate beads encapsulated with probiotic cells and/or an apple PP extract remained intact, with smooth surface and spherical shape. The co-extrusion technology used in this study allowed remarkable microencapsulation efficiency for both probiotics and apple skin PPs. The viability assay revealed that microencapsulation of PB, and more importantly co-encapsulation of PB and apple skin PP extracts, can significantly prolong the survival of PB in the milk drinks stored under chill conditions (4 °C). In contrast, the number of unencapsulated *L. acidophilus* cells present in the refrigerated milk drinks gradually declined i.e. net loss recorded as 1.1 Log CFU/mL on Day 50 of the storage under refrigeration (at 4 °C). In comparison, a loss of only 0.34 Log CFU/mL was detected when the bacterial cells were microencapsulated in the alginate beads. The co-encapsulation of probiotic cells with either ethanolic or aqueous apple skin extract resulted in smaller cell loss, i.e. 0.16 Log and 0.13 Log CFU/mL, respectively. Moreover, the microencapsulation and co-encapsulation of PB in this study provided relatively significant protection in acidified water at pH 2 for 120 min, whereas, the cell loss of unencapsulated *L. acidophilus* cells was much higher in such acidified water for 120 min. Hence, the microencapsulation of PB alone or co-encapsulation of PB and PPs in this study suggests efficient approaches for successful delivery of sufficient amounts (at least 10^6 CFU/mL) of living and active probiotic cells and PP antioxidants, which enables the efficacy claims by manufacturers of derived functional foods.

6.2 Major conclusions

It is concluded that:

- Polyphenols from apple skin can preserve the viability of probiotic bacteria in skim milk drinks
- Either the aqueous or ethanolic apple skin PP extract has the ability to enhance the adhesion of PB
- The viability of PB can be prolonged through microencapsulation of PB alone, and more preferably co-encapsulating PB and an apple skin PP extract together.
- Apple skin provides a potential source of PPs that can be used as bioactive food ingredients for dairy products containing probiotic bacteria.

This study successfully developed a polyphenol-enhanced probiotic model milk drink that contains substantial live probiotic bacterial cells for 50 days under refrigeration and acidic conditions. However, there exist limitations in this research due to the time constraints of an MSc project. The further work proposed below should be encouraged.

6.3 Future research directions

- Apply the apple skin PP extracts to other *Lactobacillus* strains and other probiotic bacteria.
- Examine the PPs from other apple cultivars and other fruits as well as their by-products, including peels, in a similar approach.
- Compare and analyse the total antioxidant activity of polyphenol-enhanced milk drinks with the milk carrying both polyphenols and probiotic bacteria.
- Develop better *in-vitro* PB adhesion assays and evaluate the adhesion efficacy using tissue-culture or an animal model.
- Examine the efficacy of co-encapsulation with apple skin polyphenols on different types of probiotic organisms, and the effectiveness of the subsequent controlled release in the gut, using an animal model.
- Study the stability of microencapsulated PB in simulated gastric fluid to demonstrate the efficacy of microencapsulation.

- Add unencapsulated PB and PPs, co-encapsulated PB and PPs to more stringent food systems with relatively higher acidity and oxygen such as yoghurt and ice cream, to examine the effects of polyphenol antioxidants on PB survival.
- Evaluate sensory characteristics of polyphenol-enhanced milk drinks carrying unencapsulated or microencapsulated PB before commercialisation of such model milk drinks.

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Appendices

Appendix I. Poster presented at the Functional Foods Symposium 2011

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John Brooks^{1*}, Dongxiao Sun-Waterhouse²

* Corresponding author: john.brooks@aut.ac.nz



Influence of the Polyphenol Extracts from Apple Skin on the Viability of Probiotic Bacteria in Model Milk Drink

1. Auckland University of Technology (AUT), School of Applied Sciences
2. Plant and Food Research

Aim

To investigate the effects of apple skin polyphenols on the survival of probiotic bacteria in a model fruit extract-enhanced milk drink.

Introduction

Functional foods containing polyphenol (PP) antioxidants and probiotic bacteria (PB) are gaining increased market leverage. Probiotic bacteria however, exhibit low viability in dairy foods during storage, due to the low pH and oxidative stress generated during processing. The low viability subsequently affects the ability of probiotic bacteria to impart their desired health benefits. PPs are well known antioxidants that can reduce the oxidative stress in food systems. Thus, it is of interest to examine the influence of added PPs on the survival of probiotic bacteria in dairy drinks.

Methods

PP extracts from apple skin were prepared using either absolute ethanol (EtOH) or citric acid infused water (Acidic H₂O), and subjected to analyses of total extractable PP content (by Folin-Ciocalteu assay, Singleton et al. 1999) and PP composition (by High Performance Liquid Chromatography, Stevenson et al. 2006). The PP extracts were then added to 12% reconstituted skim milk in the absence or presence of *Lactobacillus acidophilus* as probiotic bacteria. The obtained milk samples were stored at 4°C for 30 days, and the subsamples on Days 0, 5, 10, 15, 20, 25 and 30 were subjected to the viability assay.



Results and Discussion

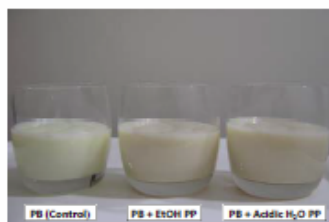


Fig 1. Probiotic (PB) milk drinks with and without added apple skin polyphenols (ethanolic PP extract, EtOH PPs, or acidic water extract, Acidic H₂O PPs)

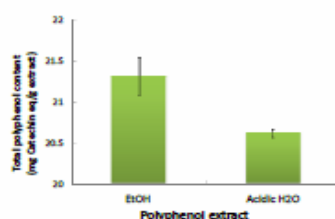


Fig 2. Total polyphenol content of apple skin extract

- Total PP content of Acidic H₂O extract was slightly lower than that of EtOH extract.

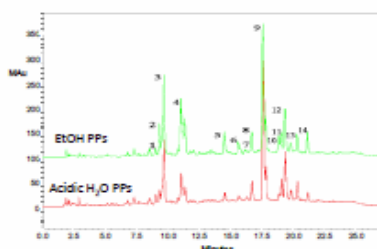


Fig 3. High performance liquid chromatography chromatograms (280 nm) for Ethanolic and acidic H₂O extracts. Peaks 1. catechin 2. caffeic acid 3. chlorogenic acid 4. epicatechin 5. p-coumaric acid 6. procyanidin trimer 7. m-coumaric acid 8. ferulic acid 9. rutin 10. o-coumaric acid or salicylic acid 11. quercetin derivative-1, 12. quercetin derivative-2 13. quercetin derivative-3 14. phloridzin

- The main species of typical apple PPs appeared in the HPLC profile of both EtOH and Acidic H₂O extracts.
- The proportion of the PPs in the two extracts differed as a result of the differences in the composition, pH and polarity of the two extracts.

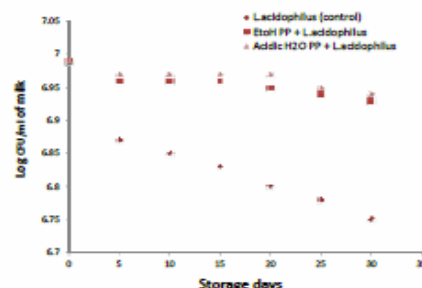


Fig 4. Influence of apple polyphenol extract on the viability of *Lactobacillus acidophilus* in model milk drinks.

- A steady loss (from 6.99 to 6.75 CFU/mL) of viability of *L. acidophilus* was detected in control milk over 30 days.
- The viability of *L. acidophilus* was significantly higher in milk drinks that were enhanced with an apple skin PP extracts (either EtOH or Acidic H₂O extract)
- The viability of *L. acidophilus* in milk enhanced with Acidic H₂O extract was marginally higher than that with EtOH PP extract especially around Day 20.

Conclusion

- Apple skin PPs can maintain the viability of probiotic bacteria in milk drink systems.
- Apple skin is a potential source of polyphenols that can be used as a bioactive food ingredient for dairy products containing probiotic bacteria.

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Ms Jing Zhou and Sandhya Wadhwa for technical assistance

Appendix II. Poster presented at the NZIFST conference 2012 and AUT postgraduate symposium 2012

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Influence of the Polyphenol Extracts from Apple Skin on the Adhesion and Viability of Probiotic Bacteria in Model Milk Drink

1. Auckland University of Technology (AUT), School of Applied Sciences
2. Plant and Food Research, Food Innovation

Aim

To investigate the effects of apple skin polyphenols on the adhesion properties and survival of probiotic bacteria in a model fruit extract-enhanced milk drink.

Abstract

This study aimed to investigate the effects of apple skin polyphenol (PP) extracts on the attachment and survival of probiotic bacteria (PB) in a model fruit extract-enhanced milk drink. Results show that the PB viability was higher in PP-enhanced milk than in milk only (control). Survival of PB in milk drinks can be prolonged by microencapsulating PB together with apple skin PPs in alginate polymer. Apple skin PP extracts also enhanced the adhesion of PB. Apple skin may be a cost-effective source of PPs for enhancing PB functionality in dairy foods.

Introduction

Probiotic therapy shows promise in the prevention or treatment of gastrointestinal (GI) disorders. However, the survival of PB in foods and their attachment to the GI tract are prerequisites for imparting desired health benefits. The health benefits of apple-derived PPs are well known. This enables apple PPs as acceptable antioxidants to consumers for reducing the oxidative stress in foods, to provide PB with an environment favourable to their survival and attachment. This study evaluated the efficacy of apple skin PPs on the attachment and survival of PB in model milk drinks.

Methods

Apple skin PP extracts were prepared using either absolute ethanol (Ethanol PP) or citric acid infused water (Aqueous PP), and then added directly, or after being microencapsulated with PB (*Lactobacillus acidophilus*) in alginate beads (Sun-Waterhouse et al., 2011), to a 12% reconstituted skim milk in the absence or presence of PB. The obtained milk samples were stored at 4 °C for 50 days, and subsamples on Days 0 to 50 were subjected to a viability assay. The influence of polyphenolic extracts on the ability of PB to adhere was estimated by Crystal violet assay (Oh et al., 2007) in aerobic and anaerobic conditions at 37 °C.



Results and Discussion

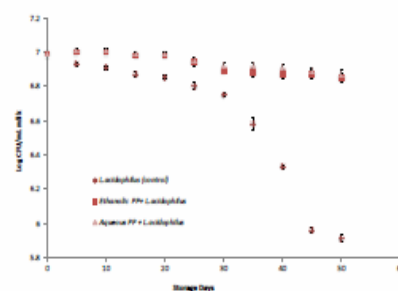


Fig 1. Influence of apple polyphenol extracts on the viability of *Lactobacillus acidophilus* in model milk drinks.

- A steady loss of viability (from 6.99 to 5.91 Log CFU/mL) of *L. acidophilus* was detected in control milk over 50 days.
- The viability of *L. acidophilus* was significantly higher in PP-enhanced milk drinks
- The viability of *L. acidophilus* in milk enhanced with aqueous PP extract was marginally higher than that with ethanolic PP extract, especially around Day 30.

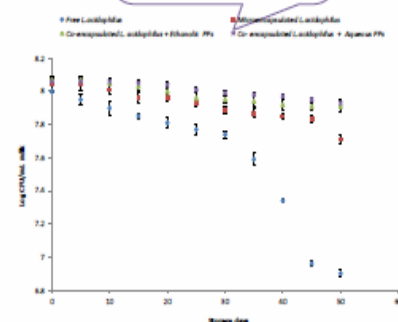
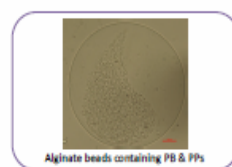


Fig 2. Influence of apple polyphenol extracts on the viability of co-encapsulated *Lactobacillus acidophilus* in milk drinks

- The viability of free (unencapsulated) PB declined gradually over 50 days.
- The viability of microencapsulated PB alone decreased (from 8.05 to 7.82 Log CFU/mL) over 50 days.
- The viability of PB microencapsulated together with either aqueous or ethanolic PP extract, was prolonged significantly in milk drinks.

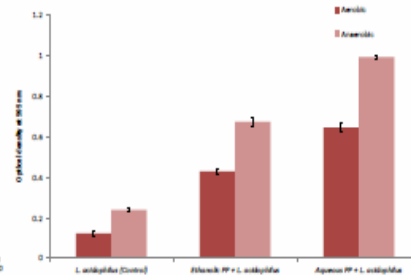


Fig 4. Influence of apple polyphenol extracts on the adhesion of *Lactobacillus acidophilus* in model milk drinks.

- Anaerobic conditions favoured significant adhesion of *L. acidophilus* compared with aerobic conditions.
- The presence of an apple skin PP extract significantly enhanced the attachment of *L. acidophilus* under both aerobic and anaerobic conditions.
- The efficiency of aqueous PP extract was significantly higher in enhancing the adhesion of *L. acidophilus* compared with that of ethanolic PP extract.

Conclusions

- Apple skin PPs can preserve the viability of probiotic bacteria in milk drink systems.
- Survival of PB in milk drinks can be prolonged by microencapsulating the bacteria with apple skin PPs.
- Apple skin PPs positively influence the adhesion properties of PB.
- Apple skin is a potential source of polyphenols that can be used as a bioactive food ingredient for dairy products containing probiotic bacteria.

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Acknowledgements

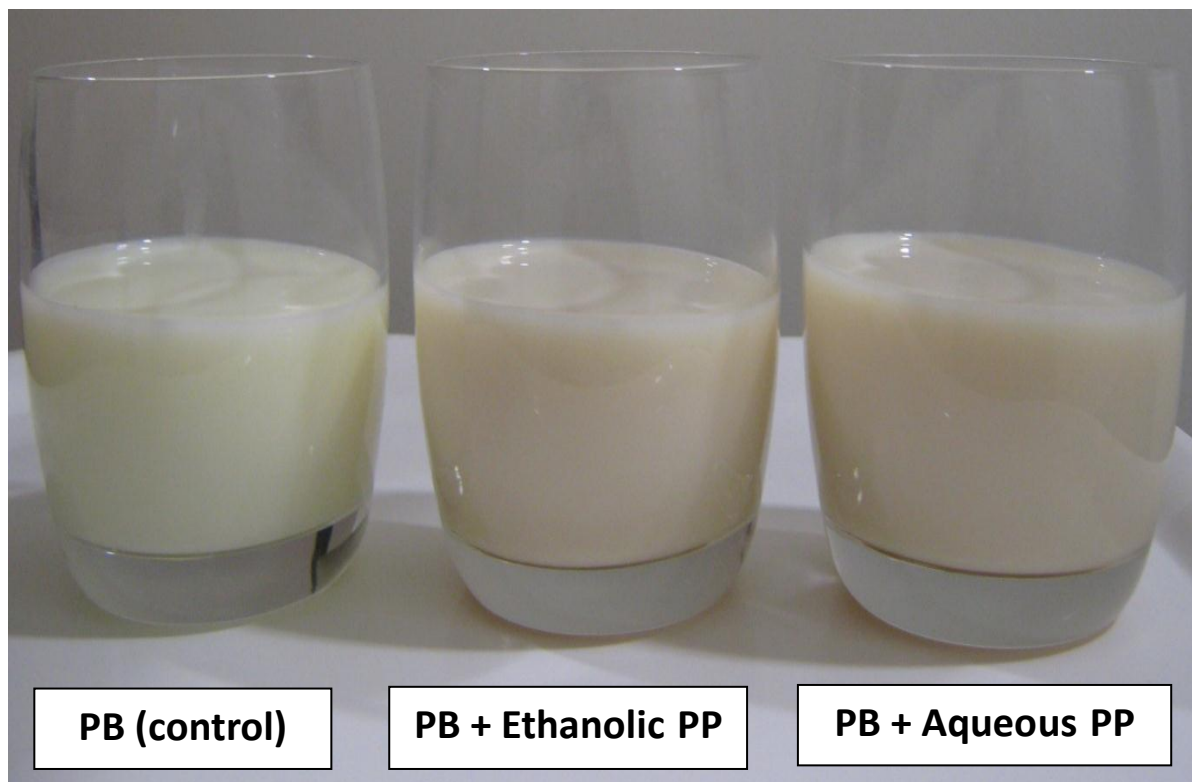
Ms Jing Zhou and Sandhya Wedhwa for technical assistance



Appendix III. The photos of the freeze-dried ethanolic and aqueous apple skin polyphenol extracts



Appendix IV. Model probiotic milk drinks enhanced with apple skin polyphenol extracts and control probiotic milk drink



Appendix V. The calibration curve of catechin standard and linear regression for TEPC analysis

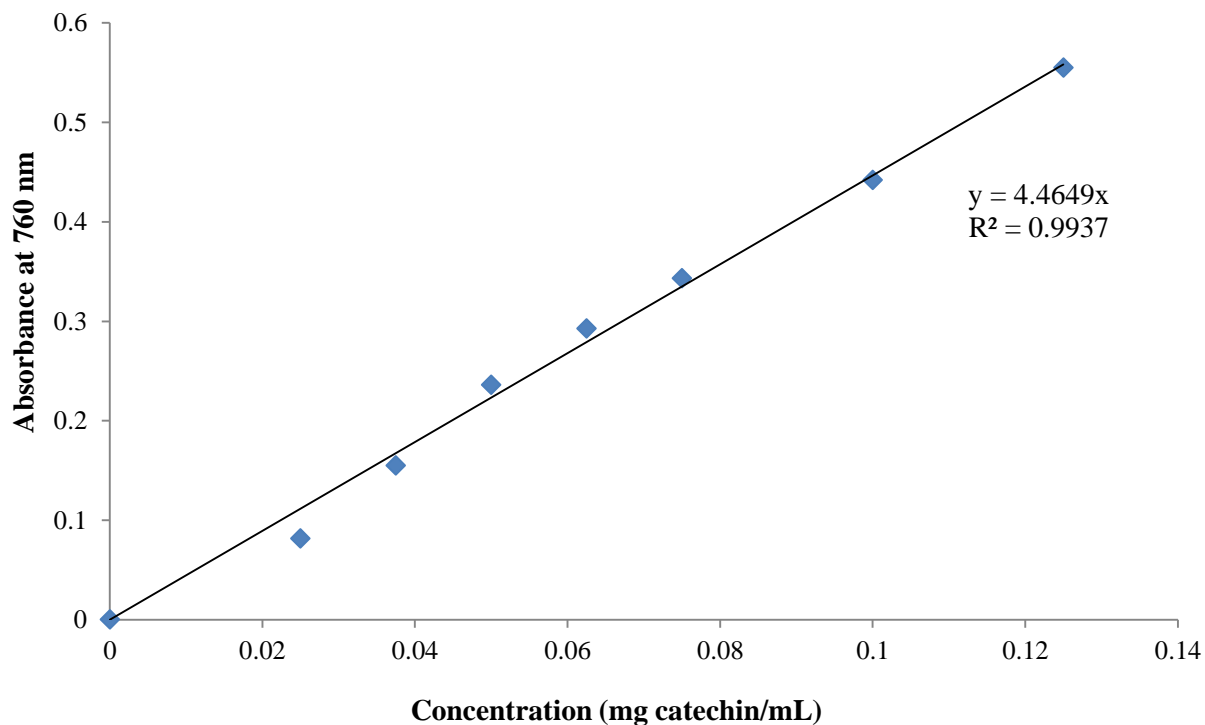


Figure 1. Calibration curve of catechin standard (mg/mL) in 25% methanol for the determination of total extractable polyphenol content by the Folin-Ciocalteu assay

Appendix VI. The calibration curve of Trolox standard and linear regression for antioxidant activity analysis

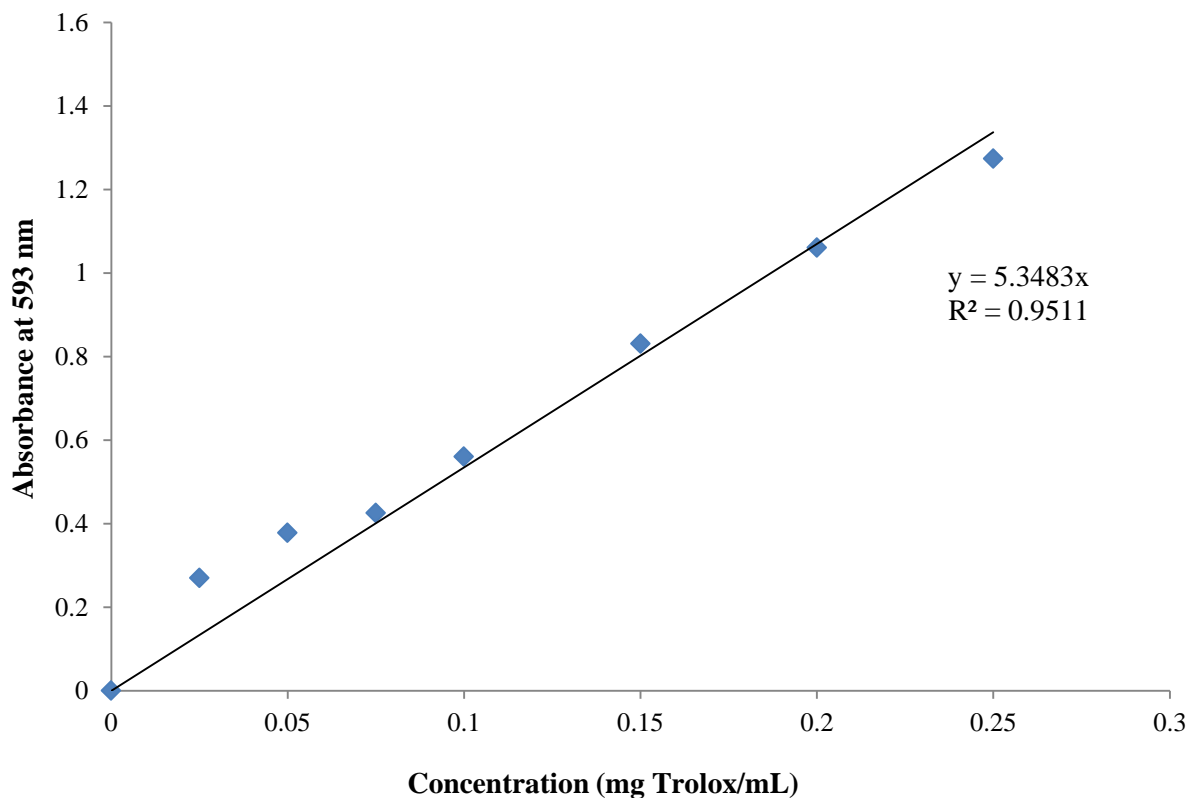


Figure 2. Calibration curve of Trolox standard (mg/mL) in 25% methanol for the determination of total antioxidant activity by the Ferric reducing antioxidant power (FRAP) assay.

Appendix VII. The calibration curve and linear regression of D-galacturonic acid standard

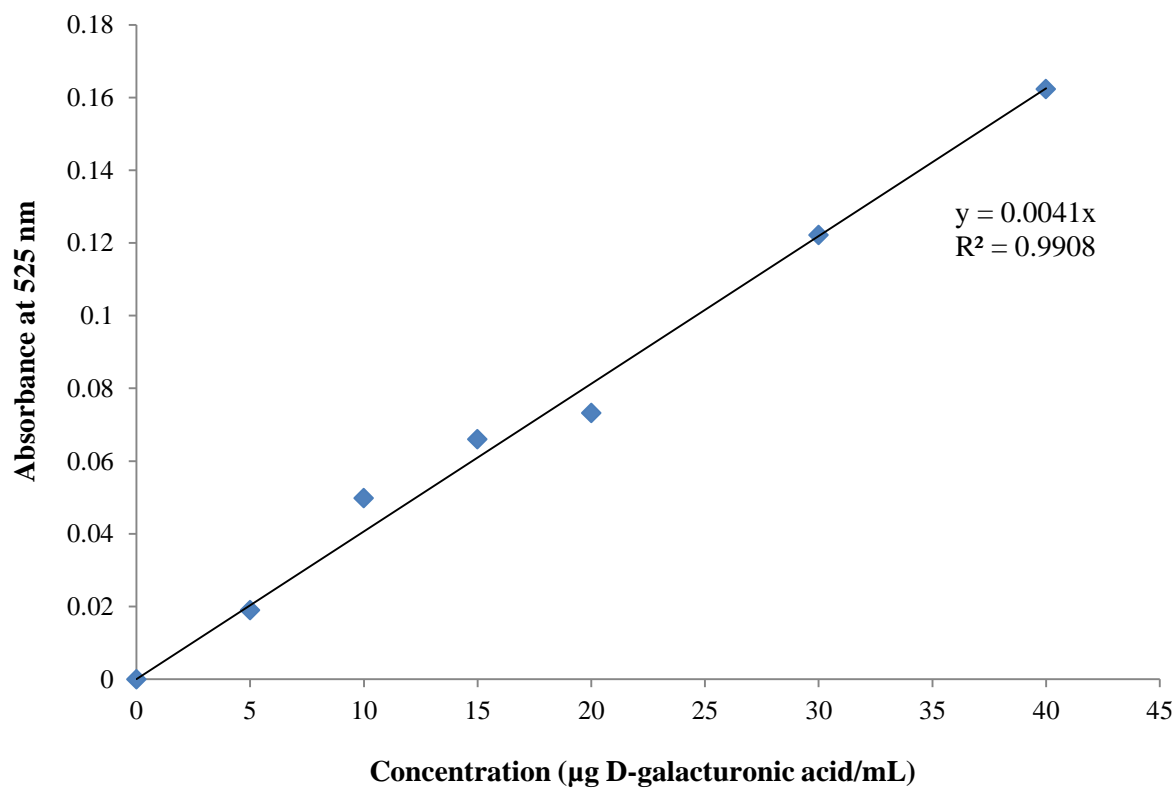


Figure 3. Calibration curve of D-galacturonic acid standard (µg/mL) for determining uronic acid content in apple skin extracts.