

**Hypocholesterolemic and Anti-hypertensive Properties of**  
***Lactobacilli* and *Bifidobacteria***

**A thesis submitted for the degree of**  
**DOCTOR OF PHILOSOPHY**

**by**

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**February 2018**



*It is my honor to dedicate this PhD thesis to*

*my parents 'Mr & Mrs Miremadí',*

*my husband 'Dr. Amír Basírat',*

*and*

*my little 'princess' BARANA*

## ABSTRACT

This project involved the development of a synbiotic dairy product and its use in human feeding trials to quantify the hypocholesterolemic and antihypertensive effects of probiotics on hypercholesterolemic men and women. To this end, eleven strains of *Lactobacilli* and three strains of *Bifidobacteria* were evaluated for their acid and bile tolerance, cholesterol removal ability and hypotensive properties *in vitro*. Our results showed that all studied strains were able to tolerate pH 2.0 for two hours. However, strains of *L. acidophilus* in general were more acid-resistant than *Bifidobacteria* with an average viable count more than  $10^7$  CFU mL<sup>-1</sup> after 2h incubation at pH 2.0. All selected strains could tolerate the bile salts, with greater tolerance towards oxgall than to taurocholic acid. The ACE inhibition was found to be significantly different among the strains ranging from 8.2% to 78.5%.

In addition to the above, all strains tested possessed varying degrees of cholesterol removal capabilities from the growth, either via medium via incorporation of cholesterol into the cellular structure, or binding of cholesterol onto cellular membrane and/or bile salt deconjugation. The affinity towards cholesterol was found to be greater in growing cells than resting or dead cells; nevertheless, this study confirmed that the latter cells could be useful as cholesterol-reducing agents in gastrointestinal tract (GIT). Fatty acid composition analysis of the harvested cells revealed that cells grown in the presence of cholesterol contained higher level of saturated fatty acids and lower level of unsaturated fatty acids than those grown in the absence of cholesterol, suggesting that cholesterol from the medium may be incorporated into the cellular membrane of probiotics. The membrane of each probiotic cell was broken up by centrifuging at  $4000 \times g$  for 20 min and fatty acid composition of harvested cells was analysed using gas chromatography.

The selected strains were also screened for their bile salt deconjugation ability and bile salt hydrolase activity (BSH). Strains of *Lactobacillus* in general showed a higher bile salt deconjugation ability and liberated a more substantial amount of cholic acid than *Bifidobacterium* strains. Substrate preference for BSH activity was more towards sodium glycocholate than sodium taurocholate, however, most strains exhibited higher total BSH activity on bile salt mixtures than to when bile salts were used individually.

Based on the above results *L. acidophilus* CSCC 2404 and *L. rhamnosus* ASCC 1520 were selected to develop a synbiotic yogurt to be used in human trials. The selected probiotics were subsequently screened in the presence of four prebiotics, sorbitol, mannitol, fructooligosaccharide, and fructooligosaccharides-enriched inulin (Synergy 1), to determine the best type and concentration of prebiotic in order to achieve maximum cholesterol removal and ACE-I activity inhibition *in vitro* and *in vivo*. Screening using factorial design showed that the combination of 1% (v/v) *L. acidophilus* CSCC 2404 and *L. rhamnosus* ASCC 1520 in the presence of 2.0% (w/v) Synergy 1 achieved the best results in synbiotic yogurt. In addition to synbiotics, there has been an increased interest in dietary sources of antioxidant polyphenols in red fruit juices such as pomegranate (*Punica granatum L.*) due to their strong antioxidant activity (AA). Therefore, to further improve the health-promoting properties of the synbiotic yogurt, different levels of pomegranate juice concentrate (PJC) (10, 12.5, 15 and 20 %) were added to yogurt milk before incubation, and the AA, antihypertensive activities and hypocholesterolemic properties of the synbiotic yogurt was evaluated during refrigerated storage for 21 days. Despite the slight adverse effect of PJC supplementation at 20% level on probiotic numbers, the probiotic population at the end of storage period was still within the acceptable range of colony- forming unit (CFU) to deliver health-promoting properties ( $> 10^6$  CFU mL<sup>-1</sup>). The resulting synbiotic yogurt showed increased polyphenol levels by

63%, AA by 94%, and angiotensin-converting-enzyme (ACE) inhibition by 75% compared to the control plain yogurt. The sensory evaluation of the PJC-enriched synbiotic yogurt revealed that by increasing the PJC level, the overall acceptability among the panellists increased significantly ( $P < 0.05$ ).

To provide evidence to support the positive effects of this PJC-supplemented synbiotic yogurt on serum lipid profile and blood pressure (BP), an 8-week parallel, double-blinded, randomised trial was designed and conducted with 48 male and female volunteers. The volunteers were aged between 30-65 years, with serum total cholesterol (TC) and triglyceride (TG) levels less than 6.2 and 2.3 mmol L<sup>-1</sup>, respectively, a body mass index (BMI) between 25 and 35 kg/m<sup>2</sup>, and willing to consuming a daily serving of 200g yogurt. Subjects were assigned to three groups; Group 1 (control) consumed the yogurt samples which only contained 0.02% (w/v) freeze-dried YC-380 (*Streptococcus thermophilus* 1275 and *L. bulgaricus* 1842), Group 2 consumed the synbiotic yogurt (Syn) containing 0.02% (w/v) freeze-dried YC-380, 0.5% (v/v) *L. acidophilus* ASCC 2404, *L. rhamnosus* ASCC 1520, and 2.0% (w/v) Synergy 1, and Group 3 consumed the same yogurt supplemented with 20% (v/v) PJC (Synp). The yogurts were produced and distributed to the participants on weekly intervals. Fasting blood samples, 3-day dietary records, anthropometric measurements and BP were collected at baseline and at the end of four and eight weeks.

Comparison of the mean differences of serum TC, TG, and high-density lipoprotein cholesterol (HDL-C) levels among the three groups confirmed a drop of 9.81% and 18.10 % in TG level and TC: HDL-C ratio ( $P < 0.05$ ) respectively, from baseline to week eight. However, no significant changes from the baseline were observed in systolic blood pressure (SBP) and diastolic blood pressure (DBP) ( $P > 0.05$ ). The *post hoc* test showed that the decrease in serum TC and LDL-C was significant for both the Syn and Synp

groups ( $P < 0.001$  for both). Consumption of Synp yogurt resulted in 19% and 23% decrease in serum TC and LDL-C levels, respectively, at the end of the study, whereas in the Syn group, these levels were only reduced by 14% and 17% respectively.

Overall, the selected probiotics *L. acidophilus* CSCC 2404 and *L. rhamnosus* ASCC 1520 proved to have the ability to tolerate acid and bile conditions and were optimised with 2.0% (w/v) Synergy 1 and 20% (v/v) PJC for maximum cholesterol removal *in vitro* and *in vivo*. Therefore, this study indicates the combined effectiveness of probiotics, prebiotics and polyphenols in ameliorating cardiovascular disease risk factors in both women and men.

## **Certificate**

**Professor Lily Stojanovska (M.Sc., PhD)**

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This is to certify that the thesis entitled “HYPOCHOLESTEROLEMIC AND ANTI-HYPERTENSIVE PROPERTIES OF *LACTOBACILLI* AND *BIFIDOBACTERIA*” submitted by **Fatemeh Miremadi** in partial fulfilment of the requirement for the award of the Doctor of Philosophy in Food Technology at Victoria University is a record of *bonafide* research work carried out by her under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

**Professor Lily Stojanovska**



**Date: 30/08/2017**

## **Declaration**

“I, Fatemeh Miremadi, declare that this thesis entitled “HYPOCHOLESTEROLEMIC AND ANTI-HYPERTENSIVE PROPERTIES OF *LACTOBACILLI* AND *BIFIDOBACTERIA*” is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

**Fatemeh Miremadi**

**Date: 30/08/2017**



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## **Acknowledgments**

My sincere thanks and gratitude go to my principal supervisor, Professor Lily Stojanovska, for her guidance, ongoing support, valuable discussions and suggestions throughout the course of study and in preparation of manuscripts. Prof. Stojanovsk's contribution to this work and the manner in which she efficiently dealt with obstacles to the progress of this project is enormously appreciated.

Also, I would like to express gratitude to my Co-supervisor Dr. Frank Sherkat (School of Sciences, RMIT University) for his extensive support and guidance throughout the study, especially at the end of my candidature with his outstanding editing skills.

In addition, I am also grateful to Prof. Nagendra Shah for his initial involvement in this project.

From depth of my heart, I would like to express my sincere gratitude, endless love and infinite respect to my parents Mr and Mrs Miremadi for their great support and prayers during my study together with my brother Dr Alireza Miremadi.

I am extremely grateful to my colleagues, especially Dr Mutamed Ayyash for his tireless support and kind friendship, and technical staff at Victoria and RMIT Universities for their help and support during my study. I would also like to acknowledge the Australian government for awarding me an Australian Postgraduate Award (APA) scholarship in 2013, which has enabled me to perform my research without any financial difficulties. The VU College of Health and Biomedicine also provided me with the financial support to broaden my scope by attending national and international conferences. And last but not least I would like to thank my husband Dr Amir Basirat for his endless support throughout this journey and my little 'princess' Barana. Her presence is a must in my life.

## List of Publications

### Peer-reviewed Publications

1. Miremadi, F., Sherkat, F., Stojanovska, L. (2016). Hypocholesterolaemic effect and anti-hypertensive properties of probiotics and prebiotics: A review. *Journal of Functional Foods*, 25: 497-510.
2. Miremadi, F., Ayyash, M., Sherkat, F., Stojanovska, L. (2014). Cholesterol reduction mechanisms and fatty acid composition of cellular membranes of probiotic *Lactobacilli* and *Bifidobacteria*. *Journal of Functional Foods*, 9: 295-305.
3. Miremadi, F., Shah, N.P. (2012). Applications of inulin and probiotics in health and nutrition: A mini review. *International Food Research Journal* 19(4): 1337-1350.

### Oral Presentations

1. **Miremadi, F.**, Sherkat, F., and Stojanovska, L. (2017). Health promoting effect of synbiotic yogurt containing pomegranate polyphenols: scientific evidence. The 10<sup>th</sup> World Congress on Nutrition & Food Sciences, 27-29 May 2017 in Osaka, Japan.
2. **Miremadi, F.**, Sherkat, F., and Stojanovska, L. (2015). Antioxidant and anti-hypertensive activities and hypocholesterolemic properties of pomegranate polyphenols in synbiotic yogurt during refrigerated storage. Post Graduate Research Student Conference, 15 July 2015, Victoria University, Melbourne, Australia.

### Poster Presentations

1. **Miremadi, F.**, Sherkat, F., and Stojanovska, L. (2014). Antioxidant and anti-hypertensive activities and hypocholesterolemic properties of pomegranate polyphenols in synbiotic yogurt during refrigerated storage. IFT, June 2014, New Orleans, USA.
2. **Miremadi, F.**, Sherkat, F., and Stojanovska, L. (2014). Cholesterol reduction mechanisms and fatty acid composition of cellular membranes of probiotic *Lactobacilli* and *Bifidobacteria*. The 47th Annual AIFST Convention, July 2014, Melbourne, Australia.

3. **Miremadi, F.**, Sherkat, F., and Stojanovska, L. (2012). Acid and bile tolerance and cholesterol removal ability of probiotic bacteria. The 45th Annual AIFST Convention, July 2012, Adelaide, Australia.

## **Awards and Grants**

1. Student travel grant for oral presentation, Osaka – Japan from College of Health and Biomedicine, Victoria University, Australia - 2017.
2. Student research grant for poster presentation, New Orleans – USA from College of Health and Biomedicine, Victoria University, Australia – 2014.
3. Australian Postgraduate Award (APA), 2013-2016.

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## **List of Abbreviations**

AA= antioxidant activity

ACE = angiotensin – converting – enzyme

AOAC = association of official analytical chemists

ANOVA = analysis of variance

ASVD = arteriosclerotic vascular disease

BMI = body mass index

BSA = bovine serum albumin

BSH = bile salt hydrolysis

BP= blood pressure

CFU = colony forming unit

CFE = cell free extract

CFS = cell free supernatant

CN = casein

CVD = cardiovascular disease

DBP = diastolic blood pressure

DP = degree of polymerization

DPPH = 2, 2-diphenyl-1-picrylhydrazyl

EA = ellagic acid

ETs = ellagitannins

EPS = exopolysaccharides

FAA = free amino acids

FCR = Folin-Ciocalteu reagent

FEI = fructooligosaccharide enriched-inulin

FOS = fructooligosaccharides

FSANZ = food standards Australia New Zealand

g = gram

GAE = gallic acid equivalents

GIT= gastro-intestinal tract

GOS = galactooligosaccharides

GSHPx = selenium-containing glutathione peroxidases

h = hour

HCE = hypercholesterolemia

HHL = hippuryl-histidyl-leucine

HDL-C = high density lipoprotein cholesterol

HP = high performance

HPLC = high performance liquid chromatography

HT= hypertension

L = litre

LAB = lactic acid bacteria

LDL-C = low density lipoprotein cholesterol

mg = milligram

min = minute

mL = millilitre

mm = millimetre

mo = month

MRS = De Man Rogosa Sharpe

NDO = non-digestible carbohydrate

NHLBI = National Heart, Lung and Blood Institute

NSLAB = non-starter lactic acid bacteria

OF = oligofructose

OPA = o-phthaldialdehyde

PAGE = polyacrylamide gel electrophoresis

PCR= polymerase chain reaction

PJC = pomegranate juice concentrate

PJ = pomegranate juice

PON1 = paraoxonase1

PSA =prostate specific antigen

PTA-SN = phosphotungstic acid soluble nitrogen

RDI = recommended daily intake

RP-HPLC = reverse phase-high performance liquid chromatography

RSM = reconstituted skim milk



s = second

SBP= systolic blood pressure

SCFA=short chain fatty acid

SN = soluble nitrogen

TC = total cholesterol

TCA = trichloroacetic acid

TFA = trifluoroacetic acid

TG = triglyceride

TN = total nitrogen

TPC = total phenolic compound

UV = ultraviolet

WBC = white blood cell

WHO = world health organization

WSE = water soluble extract

WSN = water soluble nitrogen

v/v = volume per volume

v/w = volume per weight

$\alpha$  = alpha

$\beta$  = beta

$\kappa$  = kappa

μg = microgram

° C = degree Celsius

# **1. INTRODUCTION**

Epidemiological studies have shown that higher than normal serum cholesterol is associated with the development of cardiovascular disease (CVD), which is one of the leading causes of death and disabilities in developed countries (WHO, 2015). Clinical studies have shown that for each 1 mmol L<sup>-1</sup> increase in the normal cholesterol level, the risk of coronary heart disease could increase by approximately 35%, while a small reduction in serum cholesterol of 0.1 mmol L<sup>-1</sup> can reduce the risk of coronary heart disease by 2 to 3% (Lecerf & De Lorgeril, 2011).

Hypercholesterolemia (HCE) is a condition referring to a high level of cholesterol in the bloodstream. Usually, this means that there is a high concentration of low-density lipoprotein cholesterol (LDL-C) and a low concentration of high-density lipoprotein cholesterol (HDL-C). When a higher level of LDL-C circulates within the bloodstream, it can adhere to the inner walls of arteries that feed the heart and brain causing clogging of the arteries which can lead to the development of CVD-related complications such as hypertension (HT), myocardial infarction, arteriosclerosis, angina pectoris, heart attack or stroke (Abela, 2004). The prevalence of HCE is considerably high across the populations in the developed and developing countries (Chowdhury et al., 2014).

The World Health Organization (WHO) and the American National Heart, Lung and Blood Institute (NHLBI) have classified HCE for adults (aged 18 years or above) into three main categories (WHO, 2009); total cholesterol in serum below 5.17 mmol L<sup>-1</sup> is considered normal, borderline level is between 5.17-6.18 mmol L<sup>-1</sup> and above 6.20 mmol L<sup>-1</sup> is defined as HCE. Various studies have reported a strong association between HCE and HT (Yekeen et al., 2003; Ma et al., 2009; Khalesi et al., 2014).

An increase in total blood cholesterol levels increases peripheral vascular resistance and leads to elevated blood pressure (BP) or hypertension. Hypertension has been classified into four

main categories (NHFA, 2008); normal BP is when systolic blood pressure (SBP) is less than 120 mmHg and diastolic blood pressure (DBP) is less than 80 mmHg. Prehypertension stage has been defined as a SBP of 120-139 mmHg and DBP of 80-90 mmHg, while Stage One progression HT is when SBP and DBP are elevated to 140-159 mmHg and 90-99 mmHg, respectively. Any reading of SBP above 160 mmHg and DBP higher than 100 mmHg is described as Stage Two progression HT (FitzGerald et al., 2004).

Past *in vitro* experiments and *in vivo* trials have provided evidence to support the role of probiotics in lowering serum cholesterol and improving lipid profiles, which subsequently leads to a reduced risk of HT (Kieling et al., 2002; Yeo & Liong, 2010). Various dietary approaches have been used to improve the serum cholesterol level within the population including the use of functional foods made with probiotics and prebiotics (Sindhu & Khetarpaul, 2003; Kim, 2015). Mann and Spoerry (1947) were the first to investigate the hypocholesterolemic effect of *Lactobacillus*-fermented milk in humans. The results have shown that *Lactobacilli* are not the only strains of probiotics to exhibit hypocholesterolemic effects, but *Bifidobacteria* could also cause a significant reduction in serum cholesterol. Since cholesterol absorption predominantly occurs in the intestines, intestinal microflora can have profound effects on lipid metabolism. Goel et al. (2006) have demonstrated that probiotics could lower total blood cholesterol levels and increase resistance of LDL-C to oxidation, thus leading to BP reduction.

Fuller (1989) defined probiotics as ‘live microorganisms’ which, when administered in adequate numbers in the diet, deliver health benefits to the host. Although the use of probiotics has been primarily associated with the improvement of gastro-intestinal health, recent evidence has shown that probiotics play an important role in other metabolic diseases leading to anti-hypercholesterolemic and anti-hypertensive effects. Probiotic bacteria are also able to

suppress potentially pathogenic microorganisms in the gastrointestinal tract (GIT) and improve the balance in favour of beneficial microorganisms. The mechanisms identified mainly relate to the stimulation of the host defence system, immune modulation and the competitive exclusions of pathogens (Ibrahim & Bezkorovainy, 1993; Saxelin et al., 2005). The selection of potential probiotic strains capable of performing effectively in the GIT is a significant challenge (Papadimitriou et al., 2015). Strain selection has generally been based on *in vitro* tolerance of physiologically relevant gastrointestinal conditions such as low pH and varying types and concentrations of bile salts (Gibson et al., 2000). In addition, the ability of probiotic strains to inhibit the activity of angiotensin-converting enzyme (ACE) as well as possess proteolytic (Korhonen, 2009) and bile salt hydrolysis (BSH) properties have been often included among the criteria for probiotic strain selection.

Prebiotics are non-digestible carbohydrate substrates in the diet that are the preferred foods for *Bifidobacteria* and *Lactobacilli* and result in their increased number in the large intestine (Gibson & Roberfroid, 1995). Prebiotics include fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS), glucooligosaccharides, isomaltooligosaccharides, lactosucrose, sugar alcohols, and polysaccharides (e.g. resistant and modified starches) that escape digestion in the small intestine and are fermented in the cecum and colon by the beneficial intestinal bacteria to further stimulate their numbers and activity (Gibson et al., 2000; Bielecka et al., 2002; Wolever et al., 2002). The by-products of such fermentation processes are short chain fatty acids (SCFA) mainly acetate, propionate and butyrate, which may inhibit of pathogenic bacteria (Bielecka et al., 2002), stimulate the immune system (Schley & Field, 2002), show protective effects against colon carcinogenesis (Klinder et al., 2004), improve mineral absorption (Scholz-Ahrens et al., 2002), reduce incidence of gastrointestinal diseases and improve blood lipid profile (Wolever et al., 2002; Sadrzadeh-Yeganeh et al., 2010).

In recent years, there is an increased awareness of personal health status. Often health deterioration can result from a busy lifestyle with poor choices of convenience foods, inadequate intake of dietary fibre and insufficient exercise. This has resulted in increased interest in the possibility of combining probiotics and prebiotics to produce the special group of functional foods known as ‘synbiotics’ (Williamson, 2009; Hoa & Prasad, 2013). Functional foods and drinks are products that have been enriched with added nutrients or other substances that are considered to confer more health benefits than probiotic foods alone (Granato et al., 2010; Anandharaj et al., 2014; Angmo et al., 2016). Some of the claimed health benefits include management of lactose intolerance, natural resistance to gastrointestinal infectious diseases, reduced incidence of colonic adenocarcinoma, regulated BP, reduction in serum cholesterol level and improved digestion (Kaur & Gupta, 2002; Geier et al., 2006; Liong & Shah, 2008; Khalesi et al., 2014; Ishimwe et al., 2015).

Despite various reports on the cholesterol removal ability of probiotics and prebiotics in *in vitro* experiments (Pereira & Gibson, 2002) and their hypocholesterolemic properties demonstrated by *in vivo* studies (Schaafsma et al., 1998; Delzenne & Kok, 2001), a consensus cannot be reached due to controversies raised in this area. Several *in vivo* trials linking the use of probiotic and synbiotic foods to reduce cholesterol levels have shown conflicting results. For example, Simons et al. (2006) conducted a 10-week double-blind, placebo-controlled and parallel experiment to study the effect of daily intake of four probiotic capsules, each containing  $10^9$  CFU per capsule of *L. fermentum*, on blood lipids of forty-four healthy volunteers with total serum cholesterol  $\geq 4 \text{ mmol L}^{-1}$  over a period of ten weeks. No significant changes were noted in any blood lipid markers of the study subjects. Similarly, Greany et al. (2008) also reported that daily intake of three synbiotic capsules containing  $10^9 \text{ CFU ml}^{-1}$  of *L. acidophilus* and *B. Longum*, and 10-15 mg FOS per capsule) for two months did not contribute

to any significant changes in blood lipid markers of fifty-five normocholesterolemic volunteers.

Further *in vivo* trials have failed to identify cholesterol reduction or anti-hypertensive effects due to the inherent variation in study subjects that would complicate the assessments of the mechanism involved. Therefore, the exact mechanism of cholesterol removal by probiotics is poorly understood. Some of the proposed mechanisms include incorporation of cholesterol into the cellular membrane (Kimoto et al., 2002), deconjugation of bile salts via BSH activity (Kurdi et al., 2003), and conversion of cholesterol to 5 $\beta$ - coprostanol (5 $\beta$ -cholestan - 3 $\beta$ -ol) in the intestine (Lye et al., 2010). However, studies have also found that cholesterol removal ability of probiotics is highly strain-dependent (Pereira & Gibson, 2002).

Several studies have suggested that the synbiotic combination of probiotics and prebiotics may enhance colonic bacterial composition and improve bowel function (Niness, 1999; van Zanten et al., 2012; Watson & Preedy, 2015). However, limited *in vivo* studies have specifically examined the hypocholesterolemic and anti-hypertensive effects of synbiotic products. Most of the previous studies have focused on the cholesterol-lowering and BP regulating properties of individual probiotics or prebiotics but not synergistically. This could be due to a large number of possible probiotic-prebiotic combinations as well as the complexity of evaluating the synergistic effect.

In addition to an increased demand for functional foods among the consumers due to their potential health benefits, there has been an interest in using dietary sources of antioxidant polyphenols among health-conscious people. Among red fruits, pomegranate and its derivatives have received more attention in formulation of functional foods due to their high level of total phenolic compound (TPC) and antioxidant activity (AA).



Therefore, this study was conducted in two parts; the first part was to screen the selected probiotic strains for their cholesterol removal ability and hypotensive properties, and upon this approach, to develop a synbiotic product for *in vitro* studies. The developed synbiotic yogurt was supplemented with fuctooligasaccharides-enriched inulin (FEI) and pomegranate juice concentrate (PJC) and planned for use in human feeding trials. The effect of supplementation with PJC on physicochemical and functional properties of the developed product during refrigerated storage have been investigated. The second part included human studies to determine the hypocholesterolemic and hypotensive effects of the developed synbiotic product *in vivo*. Thus, the specific objectives of this study were:

1. To evaluate the acid and bile tolerance and anti-hypertensive properties of eleven strains of *Lactobacilli* and three strains of *Bifidobacteria*,
2. To determine the mechanisms of cholesterol removal *in vitro* by *Lactobacilli* and *Bifidobacteria*,
3. To select the strains with maximum acid and bile tolerance and hypotensive properties for use in the development of synbiotic yogurt, followed by evaluating the viability of the selected strains as well as physicochemical and microbiological properties of this yogurt in the presence of FEI and different levels of PJC for maximum cholesterol removal and BP reduction during refrigerated storage,
4. To supplement the synbiotic yogurt with different levels of PJC and determine the effect of PJC on probiotic activity, and physicochemical and organoleptic properties of yogurt,
5. To evaluate the cholesterol and BP-lowering properties of the synbiotic yogurt on serum lipoprotein composition, SBP and DBP of hypercholesterolemic human volunteers.

Chapter 2 of this thesis deals with the literature review that highlighted the use of probiotics, prebiotics and synbiotics on cholesterol removal and BP-lowering, the mechanisms involved, and *in vivo* studies. Chapter 3 describes the acid and bile tolerance, and antihypertensive properties of selected lactic acid bacteria (LAB). Chapter 4 reports on the proposed cholesterol reduction mechanisms and fatty acid composition of cellular membrane of probiotic *Lactobacilli* and *Bifidobacteria*. Chapter 5 focuses on the growth optimization of the selected probiotic strains (*L. acidophilus* CSCC 2404 and *L. rhamnosus* ASCC 1520) in the presence of selected prebiotics and PJC for the maximum *in vitro* probiotic viability, cholesterol removal ability and BP-lowering properties. In addition, the effect of supplementation with different levels of PJC on probiotic activity, physicochemical and organoleptic properties was assessed. Chapter 6 reports the effects of synbiotic yogurt on lipid profile, SBP and DBP in mildly to moderately hypercholesterolemic men and women. The overall conclusions and the future directions of research are highlighted in Chapter 7 followed by references and appendices in the final chapter.

## 2. LITERATURE REVIEW

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A major part of this chapter has been published in Journal of Functional Foods: Miremadi, F., Sherkat, F., Stojanovska, L. 2016. "Hypocholesterolaemic effect and anti-hypertensive properties of probiotics and prebiotics: A review". *Journal of Functional Foods* 25, 497-510.

The World Health Organisation has predicted that by 2030, cardiovascular diseases (CVD) will be remained the leading causes of death, accounting for more than 23.6 million deaths per year around the world (WHO, 2015). Hypercholesterolemia (HCE) contributed to 45% of heart attacks in Western Europe and 35% in Central and Eastern Europe from 1999 to 2003. People affected with HCE and the associated use of cholesterol-lowering drugs may be reduced by practising dietary control and/or supplementation of probiotics and/or prebiotics.

In addition, epidemiological studies have revealed that the consumption of fruits and vegetables with relatively high phenolic content correlates with reduction of CVD, particularly heart attacks and strokes (Hertog et al., 1997; Howell & D'Souza, 2013). Therefore, in recent years, there has been increased attention by researchers, industry, and consumers on the beneficial health properties of plant-derived polyphenols such as pomegranate polyphenols. The consumption of pomegranate products leads to a significant accumulation of ellagitannins (ETs) in the large intestine, where they interact with complex gut microflora and selectively inhibit the growth of intestinal pathogens (Bialonska et al., 2009). Ellagittannins include punicalagin isomers found in the fruit, peel, bark or heartwood of pomegranate (*Punica granatum*). Ellagittannins are hydrolysed to ellagic acid (EA) under the gut physiological conditions and then gradually metabolised by intestinal microbiota to produce different types of urolithins (microflora human metabolites of ellagitannins) (Landete, 2011)

## **2.1 PROBIOTIC ORGANISMS**

Probiotics are defined as 'living microorganisms' that beneficially affect the host's health by improving its intestinal microbial balance when administered in adequate numbers in a daily diet (Fuller, 1989). They consist of lactic acid bacteria (LAB) including *Lactobacilli*, *Streptococci*, *Enterococci*, *Lactococci*, and *Bifidobacteria*, as well as other microorganisms such as *Bacillus* and *Fungi*. Many health benefits have been reported upon daily consumption

of probiotics including improved lactose intolerance (Almeida et al., 2012), increased humoral immune responses (Gourbeyre et al., 2011), increased natural resistance to infectious disease in the gastrointestinal tract (GIT) (Amaraa & Shibl, 2015), prevention of colon cancer development (Klinder et al., 2004), reducing blood cholesterol level (Gallaher et al., 2000), reducing blood pressure (Yeo & Liong, 2010), amelioration of arthritis (Songisepp et al., 2004) and facilitation of mineral absorption (Scholz-Ahrens et al., 2007). Although various probiotic bacteria have been found to possess such properties, those belonging to *Lactobacillus* and *Bifidobacterium* genera are the most common bacteria used as food adjuncts (Sanders et al., 2013; Tripathi & Giri, 2014).

### **2.1.1 Genus *Lactobacillus***

*Lactobacilli* are Gram-positive, facultative anaerobic, rod-shaped, and non-spore-forming bacteria (Gibson & Fuller, 2000; Roberfroid, 2000). They are a major part of the LAB group having the ability to convert lactose and monosaccharides to lactic acid, producing an acidic environment and inhibiting the growth of several species of harmful bacteria (Naidu et al., 1999; Makarova et al., 2006). In humans, *Lactobacilli* are normally present in the GIT as well as the female vagina, and together with *Bifidobacteria* are closely associated with the host's health, acting as an important biodefence factor in order to prevent the colonisation and proliferation of pathogenic bacteria and improve the microbial balance (Naidu et al., 1999). Species of *Lactobacillus* that have been identified as probiotics include *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. fermentum*, *L. plantarum*, and *L. reuteri*. Among LAB, *Lactobacilli* can be widely used as probiotic culture in dairy and pharmaceutical products because these species are one of the most dominant LAB that live in the human gut and enhance the overall health of humans or animals through their favourable effects on the GIT microflora balance. This may be attributed to their ability to operate using homofermentative metabolism, producing only

lactic acid from sugars and not alcohol. In addition, they are aerotolerant organisms, i.e. are not able to use oxygen for growth but tolerate its presence and therefore, could survive despite the complete absence of a respiratory chain (Sandine, 1979; Makarova et al., 2006).

*Lactobacilli* have several clinically proven health effects including reduction and prevention of diarrhoea, improvement of intestinal microbial balance, alleviation of lactose intolerance symptoms and enhancement of immune potency (McFarland, 2000; Andersson et al., 2001). Moreover, some studies have shown that certain strains of *Lactobacilli* possess anti-oxidative activity. They are able to decrease the risk of accumulation of reactive oxygen species in the host and could potentially be used in probiotic food supplements to reduce oxidative stress (Peuhkuri et al., 1996; Kullisaar et al., 2002). In a study by Kullisaar et al. (2002), it was found that daily consumption of 250 mL of milk containing *L. fermentum* ME-3 (DSM 14241) by healthy volunteers, exhibited anti-oxidative and anti-atherogenic effects. Milk fermented with *Lactobacilli* was first demonstrated to exhibit hypocholesterolemic effects in humans by Mann and Spoerry (1974), with various studies over time showing that many *Lactobacilli* strains possess a similar ability, particularly lowering the total and LDL cholesterol (Anderson & Gilliland, 1999; Sanders, 2000; Sadrzadeh-Yeganeh et al., 2010; Ejtahed et al., 2011).

Fermented foods containing *Lactobacilli* include many ancient staples, ranging from fermented milk, yogurt, cheese, wine, pickles, and sourdough bread, with many probiotic dairy products containing *Lactobacilli* (Table 2.1).

### **2.1.2 Genus Bifidobacterium**

*Bifidobacteria* are anaerobic, rod-shaped, Gram-positive bacteria that constitute a predominant part of the anaerobic flora ( $10^9$ - $10^{10}$  CFU g<sup>-1</sup>) in the adult human colon and a major part of intestinal microflora in breast-fed infants (Benno et al., 1989). Strains of *Bifidobacteria* are often used as probiotics as they are known for their resistance to bile, which

is an important attribute since the beneficial effects of probiotic bacteria must be generated in the presence of bile. It has been shown that although bile tolerance is strain-dependent, bile sensitive *Bifidobacteria* and *Lactobacilli* strains can progressively adapt to the presence of bile salts by successive subculturing in the presence of increasing concentrations of bile (Fijan, 2014). Several strains of *Bifidobacteria* are considered as important probiotics including *B. infantis*, *B. adolescentis*, *B. animalis* subsp. *animalis*, *B. animalis* subsp. *lactis*, *B. bifidum*, *B. longum*, and *B. breve*. Other health benefits have also been attributed to *Bifidobacteria*, including synthesis of vitamins and facilitation of mineral absorption (Scholz-Ahrens et al., 2007), stimulation of the immune system (Tripathi & Giri, 2014), modulation of intestinal flora (Marteau et al., 1995), improvement of constipation and alleviation of eczema symptoms in children (Sanders et al., 2013).

**Table 2.1** Biodiversity of Lactobacilli present in fermented foods

Product	Biodiversity of <i>Lactobacillus</i> spp.	Reference
<b>Dairy products</b>		
Raw milk (non- pasteurised)	<i>L. casei</i> subsp. <i>casei</i> ; <i>L. paracasei</i> subsp. <i>paracasei</i> ; <i>L. rhamnosus</i> ; <i>L. plantarum</i> ; <i>L. fermentum</i> ; <i>L. acidophilus</i>	(Quigley et al., 2013)
Cheese (hard to pasta filata cheeses)	<i>L. helveticus</i> ; <i>L. delbrueckii</i> subsp. <i>lactis</i> ; <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ; <i>L. casei</i> ; <i>L. paracasei</i> subsp. <i>paracasei</i> ; <i>L. paracasei</i> subsp. <i>tolerans</i> ; <i>L. fermentum</i> ; <i>L. curvatus</i>	(Briggiler-Marcó et al., 2007)
Cheese (goat cheese, ricotta, and mozzarella)	<i>L. bulgaricus</i> ; <i>L. casei</i> subsp. <i>casei</i> ; <i>L. helveticu</i>	(Barua et al., 2015)
Yogurt	<i>L. delbruecki</i> subsp. <i>bulgaricus</i>	(Codex alimentarius, 2003)
Fermented milk (Kefir)	<i>L. kefir</i>	(Sherkat et al., 2016)
<b>Non-dairy products</b>		
Wine	<i>L. plantarum</i> ; <i>L. brevis</i> ; <i>L. collinoides</i> ; <i>L. hilgardii</i> ; <i>L. paracasei</i>	(Testa et al., 2014)
Green table olives	<i>L. plantarum</i> ; <i>L. vermoldensis</i> sp. nov; <i>L. casei</i> ; <i>L. pentosus</i> ;	(Caggia et al., 2004)
Sourdough	<i>L. sanfranciscensis</i> ; <i>L. reuteri</i> ; <i>L. pontis</i>	(De Vuysta & Vancanneyt, 2007)
Noodles	<i>L. plantarum</i>	(Liu et al., 2011)

## 2.2 PREBIOTICS

Prebiotics are indigestible food ingredients (certain oligosaccharides and polysaccharides) that selectively stimulate the growth and activity of probiotics in the GIT (Gibson & Roberfroid, 1995). Prebiotics include fructooligosaccharides (FOS) and inulin, glucooligosaccharides, galactooligosaccharides (GOS), isomaltooligosaccharides,



lactosucrose, sugar alcohols, and polysaccharides (e.g. resistant and modified starches) that escape digestion in the small intestine and are fermented in the caecum and colon by the beneficial intestinal bacteria to further stimulate their numbers and activity (Gibson & Fuller, 2000). The by-products of such fermentation processes are short-chain fatty acids (SCFA) mainly acetate, propionate and butyrate, which may lead to inhibition of pathogenic bacteria (Bielecka et al., 2002), stimulation of the immune system (Schley & Field, 2002), prevention of colon cancer development (Klinder et al., 2004), improved mineral absorption (Scholz-Ahrens et al., 2002), reduction in incidence of gastrointestinal diseases and improved blood lipid profile (Wolever et al., 2002).

The most researched prebiotics for their possible classification as functional foods are inulin-type fructans (Watson & Preedy, 2015). They belong to a group of oligosaccharides consisting of 2 to 20 sugar units that are essentially obtained by enzymic synthesis using hydrolases and glycosyltransferases, or by partial enzymatic hydrolysis of natural long chain polysaccharides, which include natural inulin, enzymatically hydrolysed inulin, FOS, and synthetic FOS (Roberfroid, 2000).

### **2.2.1 Inulin-type fructans: chemistry and nomenclature**

Inulin-type fructans are natural components of several edible fruits and vegetables, and the average daily consumption has been estimated to be between 8 and 10 g (Mulkowski, 2013). The most common dietary sources are wheat, onion, banana, garlic, and leek (Gibson & Roberfroid, 1995). The properties of oligosaccharides used as prebiotic agents are listed in Table 2.2.

**Table 2.2** Properties of oligosaccharides used as prebiotic agent

Name	Substrate	Enzyme	Mode of production	Commercial name	General Formula
Synthetic inulin-type fructans	Sucrose	$\beta$ -fructofuranosidase	Transfructosylation	Meiologo Actilight & Nutraflora	$\alpha$ -D-Glu-(1 $\rightarrow$ 2)-[ $\beta$ -D-Fru-(1 $\rightarrow$ 2)-] <sub>n</sub> where $n = 2$ -
Natural inulin-type fructans Oligofructose-enriched Inulin (OEI; Orafit <sup>®</sup> Synergy1)	Inulin	Endoinulinase	Partial enzymatic hydrolysis	Raftilose Fibruline	$\beta$ -D-Fru-(1 $\rightarrow$ 2)-[ $\beta$ -D-Fru-(1 $\rightarrow$ 2)] <sub>n</sub> where $n = 1$ -9, and $\alpha$ -D-Glu-(1 $\rightarrow$ 2)- [ $\beta$ -D-Fru-(1 $\rightarrow$ 2)-] <sub>n</sub> where $n = 2$ -9
Fructooligosaccharides	Fructose		Transfructosylation	Oligo-Fructan Oligo-Fructose	linear chains of fructose units, linked by (2:1) bonds and often terminated by a glucose unit
Galactooligosaccharides	Lactose	$\beta$ -galactosidase	Transgalactosylation	Oligomate	$\alpha$ -D-Glu-(1 $\rightarrow$ 4)-[ $\beta$ -D-Gal-(1 $\rightarrow$ 6)-] <sub>n</sub> where $n=2$ -5
D-Sorbitol	Sucrose Glucose	Sorbitol-6-phosphate 2-dehydrogenas		D-glucitol Sorbogem Sorbo	C <sub>6</sub> H <sub>14</sub> O <sub>6</sub>
D- Mannitol	Glucose	NAD <sup>+</sup> -dependent mannitol dehydrogenase	Enzymatic hydrolysis	Aridol and Osmitol	C <sub>6</sub> H <sub>14</sub> O <sub>6</sub>
Lactoluse	Lactose		Alkali isomerisation	MLS/P/C	$\beta$ -D-Gal-(1 $\rightarrow$ 4) - $\beta$ -D-Fru
Isomalto-oligosaccharides	Starch	1- $\alpha$ -amylase $\beta$ -amylase 2- $\alpha$ -glucosidase	1-Conversion to maltose 2-Transglucosylation	Isomalto-900 Panorup Biotose & Panorich	[ $\alpha$ -D-Glu- (1 $\rightarrow$ 6)-] <sub>n</sub> where $n = 2$ -5

Macfarlane et al. (2006)

In general, fructan is a term used for any carbohydrate in which fructosyl-fructose linkages constitute the majority of the glycosidic bonds (Kolida & Gibson, 2007). Fructans are linear or branched fructose polymers, which are either  $\beta$  2  $\rightarrow$  1 linked inulins or  $\beta$  2  $\rightarrow$  6 linked levans.

Inulin is a polydisperse fructan with linear fructose polymers where oligomers are linked by  $\beta$  (2 $\rightarrow$ 1) bonds, with one glucose molecule at the end of the chain linked by  $\alpha$  (2 $\rightarrow$ 1) bond. The  $\beta$  (2 $\rightarrow$ 1) bonds prevent inulin from being digested like any typical carbohydrate, thus reducing its calorie content and resulting in dietary fibre effects (Roberfroid et al., 1998). Inulin-producing plant species are found in several monocotyledonous and dicotyledonous families, including *Liliaceae*, *Amaryllidaceae*, *Gramineae*, and *Compositae*. However, only one inulin-containing plant species, namely *Chicory Cichorium intybus* is used to produce inulin industrially.

The manufacturing process for inulin involves its extraction from chicory roots (*Cichorium intybus*). The roots are typically harvested, sliced and washed. Inulin is then extracted from the root by using a hot water diffusion process, then purified and dried (Niness, 1999). The resulting product has an average degree of polymerization (DP) of 10 to 12 units with a distribution of molecule ranging from 2 to 60 units. The finished inulin powder typically contains 6–10% sugars represented as glucose, fructose and sucrose. These are native to the chicory root; they are not added after extraction. A ‘high performance’ (HP) type of inulin has also been made available recently to the market. This product is manufactured by removing the shorter-chain molecules. HP inulin has an average DP of 25 units and a molecular distribution ranging from 11 to 60. Thus, the residual sugars as well as the oligomers have been removed. This product provides almost twice the fat mimetic characteristics of standard inulin with no sweetness (Crittenden & Planyne, 1996). DP is defined as the number of saccharides in the

fructan molecule. In chicory inulin, both Gpy-Fn (a-D-glucopyranosyl-[b-D-fructofuranosyl] n21-b-D-fructofuranoside) compounds are considered to be included under the same nomenclature, and the number of fructose units varies from 2 to >70.

Fructooligosaccharides, also called oligofructose (OF), are derived from chicory in much the same manner as inulin. The major difference is the addition of a hydrolysis step after extraction. The chemical structure of FOS consists of a chain of 2 to 10 fructose units with a terminal glucose unit linked by glycosidic bonds (Niness, 1999). Common types of FOS include 'neosugar', which is a mixture of three oligosaccharides of different lengths, such as kestose (GF<sub>2</sub>), nystose (GF<sub>3</sub>) and β-fructosylnystose (GF<sub>4</sub>). FOS derived from chicory contains both fructose (F<sub>m</sub>) and fructose chains with terminal glucose units while synthesized FOS contains only fructose chains with terminal glucose units, (GF<sub>n</sub>). Both types of FOS contain β (2→1) bonds between the fructose molecules, and have the same prebiotic effects (Roberfroid et al., 1998).

The differences in chain length between inulin and FOS account for their distinctly different functional attributes. Due to its longer chain length, inulin is less soluble than FOS and has the ability to form inulin microcrystals when sheared in water or milk, form a smooth creamy texture and provide a fat-like mouthfeel. Inulin has been used successfully to replace fat in table spreads, baked goods, fillings, dairy products, frozen desserts and dressings (Crittenden & Planyne, 1996). Fructooligosaccharides are composed of shorter-chain oligomers and possess functional qualities similar to sugar or glucose syrup. They are actually more soluble than sucrose and provide 30-50% of the sweetness of table sugar. FOS provides crispness to low-fat cookies, and act as a binder in nutritional or granola bars, in much the same way as sugar, but with the added benefits of fewer calories, fibre enrichment and other nutritional properties. FOS is often used in combination with high intensity sweeteners to replace sugar,

provide a well-balanced sweetness profile and cover aspartame aftertaste (Weidmann & Jager, 1997).

Both inulin and FOS are used worldwide to add fibre to food products. Unlike other fibres, they have no “off flavours” and may be used to add fibre without contributing viscosity. These properties allow the formulation of high fibre foods that look and taste like standard food formulations. In essence, it is an undetectable way to add fibre to foods. Consequently, FOS is commonly used in cereals, fruit preparations for yogurt, frozen desserts, cookies and nutritional dairy products. The nutritional properties of inulin and FOS are similar; thus the decision to formulate with inulin instead of FOS largely depends on the desired attributes of the finished product. For example, the use of HP inulin would prove to be the method of choice when formulating a low-fat table spread that has a creamy, fat-like mouthfeel with no added sweetness. Conversely, when formulating a low-calorie fruit preparation for yogurts using high intensity sweeteners, FOS could enhance the fruit flavour, balance the sweetness profile and mask any undesirable aftertaste. Another added benefit of FOS that is often capitalized on in yogurt is the prebiotic effect, which may enhance the probiotic properties of cultures typically added to yogurt (Niness, 1999).

Inulin and FOS are not absorbed or degraded in the upper human GIT so they enter the colon intact and are fermented by the resident microbiota. FOS (oligofructose) is a simpler fibre molecule and completely metabolised in the proximal part of the colon, whereas the colon fermentation of inulin, which is a more complex molecule, usually occurs in the distal part of the colon. The 30/70 mixture (wt: wt) of oligofructose and inulin is called oligofructose-enriched inulin (OEI). This mixture provides a synergy whereby the mixture of the two has been found to be more effective in producing beneficial results than either one alone. Oligofructose-enriched inulin is an effective prebiotic for the whole length of the colon, and

this synergy has more nutritional and health benefits than its components (Roberfroid, 2007). Moreover, it has been shown to shift the composition of the gut microbiota toward higher concentrations of *Bifidobacteria* and *Lactobacilli*. The intake of 5 to 8 g d<sup>-1</sup> of inulin-type fructans has been shown to have a positive effect on gut microbiota (Dehghan et al., 2014).

### **2.2.2 Inulin-type fructans and the concept of dietary fibre**

Roberfroid (2000) defined the five basic attributes of dietary fibre as:

1. Components of edible plant cell;
2. Carbohydrates (both oligosaccharides and polysaccharides);
3. Human hydrolysis- resistant (mammalian) alimentary enzymes;
4. Non-absorbable in the small intestine;
5. Fermentable (partial or total) by the colonic bacteria.

Inulin-type fructans fit the above definitions as they are plant carbohydrates that, because of the  $\beta$  (2  $\rightarrow$  1) configuration of the fructosyl-fructose glycosidic linkages, resist digestion in the upper GIT but are quantitatively fermented in the colon. They are thus undoubtedly part of the dietary fibre complex, and they must be labelled as dietary fibre on consumer food products. However, because of their specific colonic fermentation end products (acetate, butyrate propionate, lactate and gases), inulin-type fructans differ from non-fermentable dietary fibres such as bulking fibres (e.g. psyllium, cellulose and hemicellulose). Therefore, they may contribute in a significant way to a well-balanced diet not just by increasing the fibre content and diversity but also by specifically affecting the composition of intestinal microflora, mucosal functions, endocrine activities, mineral absorption and improving the systemic functions especially digestive function, lipid and glucose metabolism, as well as the immune system and immune functions (Guarner, 2005; Anderson et al., 2009; Simpson & Campbell, 2015)

### 2.2.3 The prebiotic properties of inulin-type fructans

Inulin and oligofructose are the most studied and well-established prebiotics. They escape digestion in the upper GIT and reach the large intestine virtually intact, where they are quantitatively fermented in the proximal and distal parts of the colon, respectively, and act as prebiotics. Indeed, in the studies investigating the effects of inulin and oligofructose on the human gut microbiota, a selective growth stimulation of *Bifidobacteria* and *Lactobacilli* has been reported (Tuohy et al., 2001; Guigoz et al., 2002; Kolida & Gibson, 2007; Dewulf et al., 2012). Bacterial flora colonising the particular surfaces of the large intestine play specific roles in the protection of the intestinal lumen through: 1) changing the composition of the intestinal environment, and 2) influencing the miscellaneous functions of the epithelium (Macfarlane et al., 1999).

Previous studies indicated that partially ingested inulin and fully ingested FOS, are fermented by colonic microflora. The major products of this fermentation process are SCFAs, the gases hydrogen and carbon dioxide, and bacterial cell mass (Knudsen & Hesso, 1995; Kolida & Gibson, 2007). *Bifidobacteria* constitute a significant portion of the colonic microflora and have beneficial effects on their host. (Roberfroid, 2007). In addition, *Bifidobacteria* produce lactic and acetic acids which acidify the large intestine and act as a potent antimicrobial substance to putrefactive microorganisms (Rossi et al., 2005).

Short chain fatty acids are absorbed by the perfused human colon in a concentration-dependent manner and are the major respiratory fuels for colonocytes, supplying up to 60 to 70 % of their energy needs. They also stimulate the growth of colorectal mucosal cells, retard mucosal atrophy, and decrease the risk of malignant transformation in the colon. Butyrate has been shown to be particularly effective in decreasing the risk of malignant transformation of the colon (Rossi et al., 2005).

Swanson et al. (2002) found that adding FOS to canine diets increased lactate production by *Bifidobacteria* in their gut and decreased putrefactive compounds such as indoles and phenols. They also found that the ingestion of FOS increased the numbers of aerobes and *Lactobacilli* in the guts of dogs. *Bifidobacterial* numbers increased followed by a decrease in the *Clostridia perfringens* concentrations, a positive indicator of colon health. Dramatic positive shifts in the composition of colonic microflora have been shown through human studies at doses between 5 to 20 g day<sup>-1</sup> over a 15-day period (Gibson & Roberfroid, 1995). This is contributed to the possession of a competitive  $\beta$ -fructosidase enzyme by bacteria that catalysed the cleavage of glycosidic linkages in FOS to release fructose for metabolism via the “bifidus” pathway (Rossi et al., 2005). The bifidogenic effects of inulin and FOS are independent of chain length and terminal units whether fructose or glucose (Roberfroid et al., 1998). *Bifidobacteria* were found to have a preference for FOS over glucose. However, very little information is available on the uptake of carbohydrates by *Bifidobacteria*, although it appears that the substrate transport systems may be more efficient for dimeric and oligomeric carbohydrates (Gibson & Wang, 1994).

## 2.3 SYNBIOTICS

In recent years, due to a busy lifestyle with poor choices of convenience foods, inadequate intake of dietary fibre and insufficient exercise; interest has risen in the possibility of combining probiotics and prebiotics to produce the special group of functional foods known as ‘synbiotics’ that confer more health benefits than probiotic foods alone (Bielecka et al., 2002). Kieling et al. (2002) evaluated the hypocholesterolemic effect of a synbiotic yogurt (containing *L. acidophilus* 145, *B. longum* 913 and oligofructose) in humans over six months. In this Randomised, placebo-controlled and cross-over study, daily consumption of 300g of synbiotic yogurt by twenty-nine women significantly decreased their LDL-C: HDL-C ratio from 3.24 to



2.48 ( $P=0.001$ ). Subsequently, Liong et al. (2007) reported that the daily administration of a synbiotic capsule (containing 1.00 g freeze-dried *L. acidophilus* ATCC 4962; 1.25 g FOS; 1.56 g mannitol and 2.20 g inulin) in the ration of twenty-four hyper-cholesterolemic male pigs for eight weeks resulted in a significant drop in their plasma total cholesterol level, from  $8.98 \pm 0.23$  to  $6.81 \pm 0.23$  ( $P < 0.001$ ), the LDL cholesterol level from  $6.62 \pm 0.25$  to  $5.58 \pm 0.25$  ( $P < 0.045$ ), and triglyceride (TG) level from  $7.0 \pm 0.40$  to  $4.81 \pm 0.40$  ( $P < 0.001$ ). Although the combination of probiotic and prebiotics was reported to synergistically reduce serum cholesterol levels, still more clinical trials are required on a suitable combination of synbiotics that specifically target reduction of serum cholesterol levels in animals and humans. This could be due to the large number of possible probiotic-prebiotic combinations as well as the complexity of evaluating the synergistic effect.

#### **2.4 SURVIVABILITY OF PROBIOTICS UNDER ACIDIC AND BILE CONDITIONS**

The dominant food vehicles for probiotics remain to be fermented milk products such as yogurt, which provide a relatively low-pH environment for the bacteria. Before, they begin their journey through the GIT, the bacteria pass through the mouth where the temperature and pH is suitable to their survival and the residence time is short. When probiotic bacteria reach the stomach they are exposed to the extreme acidic conditions and the activity of the digestive enzymes (Fuller, 1992; Gibson & Fuller, 2000). The time for the ingested food to be released from the stomach is reported to be approximately ninety minutes to two hours and therefore, the probiotic bacteria must resist the stressful conditions of the stomach and upper intestine. Cellular stress begins in the stomach, with a pH as low as 1.5 (Guarner & Schaafsma, 1998; Gibson et al., 2000) and the upper intestinal tract where bile is secreted into the gut (Lian et al., 2003). Therefore, probiotic bacteria should be able to survive acidic stress for at least ninety

minutes and tolerate bile, so they can attach to the intestinal epithelium and grow in the lower intestinal tract before they can start providing any health benefits. Hence, acid and bile tolerances are the first properties screened when selecting probiotic strains. *In vitro* screening tests for selection of acid and bile tolerant strains can readily be applied to ensure the quality of probiotic cultures during manufacture, and shelf life of the product (Pereira & Gibson, 2002; Lye et al., 2010). However, the quantitative extrapolation of probiotic performance *in vivo* has been difficult, due to intra-species variations between potential probiotic strains. In addition, *in vivo* experiments would also involve environmental factors that affect culture growth and induce stress, causing changes in culture performance. Thus, *in vitro* experiments have often been used and efforts have been made to mimic the *in vivo* systems in the best possible ways. Since the pH of the stomach is known to fluctuate, Draser et al. (1969) adjusted the pH of the gastric juice taken from healthy subjects prior to use, and reported that most *Lactobacillus* strains showed the ability to survive and reached the intestinal environment to function effectively. *Bifidobacterium* strains however, proved less resistant to human gastric fluids.

#### **2.4.1 Bile synthesis, storage and secretion**

Bile is a yellow/green aqueous solution of organic and inorganic compounds and composed of bile acids and salts, phospholipids, cholesterol, pigments, water, and electrolyte chemicals that keep the total solution slightly alkaline (with a pH of about 7 to 8). Immunoglobulin A and mucus are secreted into bile to prevent bacterial growth (Begley et al., 2005). Many endogenous substances including water-soluble vitamins (vitamin B12, folic acid and pyridoxine), all estrogenic steroids, progesterone, testosterone, corticosteroids and essential trace metals. Bile is generally isotonic with plasma with an osmolality of approximately 300 mOsm/kg that is primarily attributable to the osmotic activity of the inorganic ion (Johnson, 1998).

Bile is synthesised in the pericentral hepatocytes of the liver and is secreted into thin channels called bile canaliculi. These canaliculi drain into bile ducts that merge to form hepatic ducts. Bile leaves the liver through the common hepatic duct that is joined by the cystic duct from the gallbladder to form the common bile duct. Bile leaving this duct enters the duodenum at a junction regulated by a sphincter termed the sphincter of Oddi. Then, bile is diverted to the gallbladder where it is concentrated approximately five- to ten-fold. Water and electrolytes are removed and bile is acidified by  $\text{Na}^+/\text{H}^+$  exchange (Begley et al., 2005). The gallbladder is not essential for bile secretion but facilitates its storage in preparation for fat digestion. In fact, only half the hepatic bile enters the gallbladder for concentration and storage, the other half bypasses the gallbladder to enter the duodenum and undergoes continuous enterohepatic cycling. When chyme from an ingested meal enters the small intestine, acid and partially digested fats stimulate secretion of secretin and cholecystokinin. These enteric hormones are important for the secretion and flow of bile. Secretin stimulates biliary duct cells to secrete bicarbonate and water to expand the volume of bile. Cholecystokinin (cholecysto = gallbladder, kinin = movement) stimulates contractions of the gallbladder and the common bile duct (Johnson, 1998; Begley et al., 2005). As a result, the gallbladder contracts, the sphincter of Oddi relaxes, and up to 80% of the gallbladder contents are discharged into the duodenum in an exponential fashion.

#### **2.4.2 Bile acids**

Bile acids are steroid acids found predominantly in the bile of mammals and other vertebrates. Different molecular forms of bile acids can be synthesized in the liver by different species (Hofmann et al., 2010). Bile acids are conjugated with taurine or glycine in the liver, forming primary bile acids (Russell, 2003). The sodium and potassium salts of bile acids are called bile salts. The primary bile acids are those synthesized by the liver whereas the secondary

bile acids result from bacterial actions in the colon (Ruiz et al., 2013). In humans, taurocholic acid and glycocholic acid (cholic acid derivatives), taurochenodeoxycholic acid and glycochenodeoxycholic acid (chenodeoxycholic acid derivatives) are the major bile salts in bile and are roughly equal in concentration (Rossi et al., 2005).

Bile acids compose approximately 80% of the organic compounds in bile (others are phospholipids and cholesterol) and an increased secretion of bile acids produces an increase in bile flow. The main function of bile acids is to allow digestion of dietary fats and oils by acting as a surfactant that emulsifies them into micelles, allowing them to be colloidally suspended in the chyme before further processing (Fiorucci et al., 2009)

## **2.5 CLINICAL SIGNIFICANCE**

The World Health Organization (WHO) estimates 17.5 million deaths every year from CVD, particularly heart attacks and strokes, a number that is expected to grow to more than 23.6 million by 2030 (WHO, 2015). A substantial number of these fatalities can be attributed to lipid profile abnormalities, which triples the risk of heart attack in people with HCE, compared to those with normal blood lipid profiles (Lecerf & De Lorgeril, 2011). Unhealthy diets such as those high in salt and fat, especially saturated fats, and low in complex carbohydrates, fruits and vegetables, lead to increased risk of cardiovascular diseases (Torpy, 2009). Therefore, various dietary approaches have been employed to alleviate HCE at the population level, including the use of probiotics and prebiotics in the development of functional foods.

Hypercholesterolemia (HCE) is a condition which refers to an extremely high level of cholesterol in the bloodstream. Usually this means that there is a high concentration of LDL cholesterol and a low concentration of HDL-cholesterol. When too much LDL cholesterol circulates within the bloodstream, it can stick to the inner walls of arteries that feed the heart

and brain, causing stenosis of the arteries and a series of cardiovascular diseases such as hypertension, myocardial infarction, arteriosclerosis, angina pectoris, myocardial infarction (MI) or stroke (Chowdhury et al., 2014). The prevalence of HCE is considerably high across populations in the developed and developing countries regardless of economic, social, and health status (Yekeen et al., 2003). Total blood cholesterol levels exceeding  $6.4 \text{ mmol L}^{-1}$  increase peripheral vascular resistance and therefore lead to elevated BP.

The WHO and the Australian National Heart Foundation and High Blood Pressure Research Council (NHFRC) have classified HCE for adults (aged 18 years or above) into three main categories (WHO, 2009); total cholesterol in serum below  $5.17 \text{ mmol L}^{-1}$  is considered normal, borderline level is between  $5.17\text{-}6.18 \text{ mmol L}^{-1}$  and above  $6.20 \text{ mmol L}^{-1}$  is defined as HCE. Different studies have reported a strong association between HCE and hypertension (HT). Hypertension has been classified into four main categories. Normal blood pressure (BP) is defined as a systolic blood pressure (SBP) of less than 120 mm Hg and a diastolic blood pressure (DBP) of less than 80 mm Hg. Prehypertension stage has been defined as an SBP of 120-139 mm Hg and DBP of 80-90 mm Hg, while stage one progression hypertension is SBP of 140-159 mm Hg and DBP of 90-99 mm Hg. Any reading of SBP above 160 mm Hg and DBP higher than 100 mm Hg is described as stage two progression hypertension (FitzGerald et al., 2004).

Atherosclerosis (also known as art arteriosclerotic vascular disease or ASVD) is a specific form of arteriosclerosis in which an artery wall thickens as a result of invasion and accumulation of white blood cells (WBC) which, is termed 'fatty streaks' (Hansson & Hermanson, 2011). These accumulations contain both living, active WBCs and remnants of dead cells, including fat, cholesterol, triglycerides and calcium. The 'fatty streaks' reduce the elasticity of the artery walls. However, they don't affect blood flow for decades because the

artery muscular wall enlarges at the location of plaque. When these fatty plaques rupture, they form a thrombus (blood clot) that can further limit, or even block the flow of oxygen-rich blood to organs and other parts of the body (Prasad et al., 2014).

Atherosclerosis can occur in arteries anywhere in the body but is most serious when it leads to a reduced or blocked blood supply to the heart or to the brain. If it occurs in one of the two main coronary arteries that supply blood to the heart, this results in a heart attack. When thrombosis occurs in one of the arteries to the brain, it causes a stroke.

Atherosclerosis starts when the endothelium becomes damaged. It is usually caused by risk factors such as high blood pressure, smoking, or high cholesterol. When the endothelium is damaged, LDL cholesterol begins to accumulate in the artery wall. To combat this, the body sends WBC, such as macrophages to clean up the accumulated cholesterol molecules. If these cells get stuck at the affected site the result is a plaque build-up, mainly made up of LDL cholesterol, and macrophage white blood cells. As atherosclerosis progresses, the plaque gets bigger and it may create a blockage (McNeal et al., 2010; Prasad et al., 2014).

This association between HCE and HT could be due to over-activation of the sympathetic nervous system by leptin (Agata et al., 1997; Ma et al., 2009). Leptin is a key neuroendocrine hormone regulating food intake, metabolism, and fat accumulation, which alters lipid profiles, increases peripheral vasoconstriction as well as renal tubular reabsorption leading to increased blood pressure (Ma et al., 2009). Hypertension has also been associated with renin activity, an enzyme generated from the inactive precursor prorenin, that participates in the body's rennin-angiotensin system (Inagami, 1992; Drenjancevic-Peric et al., 2011). This enzyme circulates in the blood stream and hydrolyses angiotensinogen (secreted from the liver) into the peptide angiotensin I which is further converted into angiotensin II by an angiotensin-converting enzyme. Angiotensin II causes vasoconstriction, induces the release of aldosterone, increases

the sodium concentration, causes fluid retention, which increases the pressure exerted by the blood against blood vessel walls, resulting in high blood pressure (Inagami, 1992; Drenjancevic-Peric et al., 2011).

Hypertension is often associated with blood lipid abnormality and patients with hypertension frequently have a lower level of HDL-C and a higher level of LDL-C (Geol, et al., 2006). Many drugs such as statins have been developed to reduce the level of LDL cholesterol in hypercholesterolemic patients. However, the undesirable side effects of these compounds including headache, abdominal pain and constipation were observed and have caused concerns about their long-term therapeutic use. Therefore, non-pharmaceutical approaches including dietary interventions have been employed.

### **2.5.1 Dietary approaches in HCE management**

A number of *in vitro* experiments and *in vivo* trials have provided evidence to support the role of probiotics, prebiotics and phytosterols in lowering serum cholesterol and improving lipid profiles, which subsequently lead to control of BP.

Many animal and human trials to evaluate the hypocholesterolemic effects of probiotics and prebiotics have shown promising results. The hypocholesterolemic effects of probiotic bacteria specifically *Lactobacilli* and *Bifidobacteria* was first illustrated by Mann and Spoerry (1974). They proposed that cholesterol absorption occurs in the intestines, therefore intestinal microflora have a predominant role in lipid metabolism. Animal studies have demonstrated that probiotics could improve lipid abnormalities by lowering the blood cholesterol level and increasing the resistance of LDL cholesterol to oxidation, and thereby reduce blood pressure. Recent studies covering evidences and controversies in HCE management are summarized in Table 2.4

**Table 2.3** *In vitro* studies on the hypocholesterolemic effects of probiotic strains

Probiotic or Synbiotics	Study plan	Effects	References
142 isolates of LAB strains	1. Cholesterol assimilation 2. BSH activity	Cholesterol removal from the media into the cellular membrane of probiotic bacteria during growth and production of BSH	(Shehata et al., 2016)
5 strains of <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> isolated from home-made yogurt	Incorporation of cholesterol into the probiotic cellular membrane	Cholesterol removal	(Tok & Aslim, 2010)
15 strains of <i>Lactobacilli</i> and <i>Bifidobacteria</i>	Cholesterol assimilation	Cholesterol removal from the media into the cellular membrane of probiotic bacteria during growth	(Lye et al., 2010)
8 <i>L. plantarum</i> and 5 <i>L. paracasei</i> strains isolated from cheese	Cholesterol assimilation	Cholesterol removal from the media into the cellular membrane of probiotic bacteria during growth	(Belviso et al., 2009)
7 strains of <i>L. casei</i> and 4 strains of <i>L. acidophilus</i>	1. BSH activity 2. Co-precipitation of cholesterol with deconjugated bile salt	Cholesterol removal via cholesterol co-precipitation and production of BSH	(Liong & Shah, 2005)
7 strains of <i>Lactococci</i>	Incorporation of cholesterol into the probiotic cellular membrane	Cholesterol removal	(Kimoto et al., 2002)
6 strains of <i>L. acidophilus</i>	Cholesterol assimilation	Cholesterol removal via assimilation of the cholesterol by <i>L. acidophilus</i> cells or the cholesterol attachment to the surface of <i>L. acidophilus</i> cells	(Lin & Chen, 2000)
Human intestinal bacteria, collection of yeast from the intestine and cultures of <i>Pseudomonas aeruginosa</i>	Bile salt deconjugation	No significant cholesterol reduction	(Dambekodi & Gilliland, 1998)
<i>B. longum</i> BB 536, <i>B. infantis</i> ATCC 15697, <i>B. breve</i> ATCC 15700 <i>B. breve</i> ATCC 15698, <i>B. animalis</i> ATCC 25527	Deconjugation of bile salts	Cholesterol removal by probiotic bacteria via the production of BSH and hydrolysing the glycin- or taurin- conjugated bile salts into amino acid residues and free bile salts	(Gilliland et al., 1985)



**Table 2.4** *In vivo* studies on the controversial hypocholesterolemic effects of probiotics and synbiotics

Probiotics or Synbiotics	Study design	Animals/Subjects	Dose, duration of the study	Effects	References
<b>Animal studies</b>					
<i>L. plantarum</i> KCTC3928	Randomised, placebo-controlled, parallel study	21 six-week-old male hypercholesterolemic mice	3 × 7 mice: Control group: No probiotic; Test groups: 1 × 10 <sup>9</sup> CFU mL <sup>-1</sup> live (T1) or dead (T2) cells, administered orally of 4 week weeks	TC: 33% decrease LDL: 42% decrease TG: 32% decrease HDL: 35% increase (P < 0.05)	(Jeun et al., 2010)
<i>L. plantarum</i> CK 102	Randomised, placebo-controlled, parallel study	32 x 5-week-old male hypercholesterolemic Sprague-Dawley (SD) rats, BW :129 ± 1 g	Daily intake of 5.0 × 10 <sup>7</sup> CFU mL <sup>-1</sup> of probiotic for 6 weeks	TC drop: 27.9% LDL drop: 28.7% TG drop: 61.6% (P < 0.05)	(Ha et al., 2006)
<i>L. casei</i> NCDC-19 and <i>Saccharomyces boulardii</i>	Randomised, placebo-controlled, parallel study	20 young Swiss mice	A mixture of barley flour, sprouted green gram paste, milk powder, tomato pulp and 1% cholesterol; fermented with 10 <sup>9</sup> CFU g <sup>-1</sup> of each probiotic; fed for 42 days	TC:19% decrease LDL :37% decrease (P < 0.05)	(Sindhu & Khetarpaul, 2003)
<i>L. acidophilus</i> ATCC 43121	Randomised, double-blind, placebo controlled study	33 hypercholesterolemic pigs	A ration containing 2.5 × 10 <sup>11</sup> CFU g <sup>-1</sup> fed for 15 days	TC: 11.8% decrease (P < 0.05)	(De Rodas et al., 1996)
<b>Human studies</b>					
<i>B. longum</i> , <i>B. infantis</i> , <i>B. breve</i> , <i>L. acidophilus</i> , <i>L. paracasei</i> , <i>L. delbrueckii</i> subsp. <i>L. plantarum</i> , & <i>S. salivarius</i> subsp.	Randomised, placebo-controlled study	60 overweight healthy adults, mean age:49 years	Capsules each containing 112.5 × 10 <sup>9</sup> CFU ml <sup>-1</sup> probiotic (P) or probiotics plus omega-3 (O3P): 180 mg omega-3 Eicosapentaenoic acid (EPA) and 120 mg docosahexaenoic acid (DHA) for 6 weeks	LDL drop by 7.04% in (P) group and 10.7% in (O3P) group, HDL rise in (O3P) group resulting in 6.7% increase in TC	(Rajkumar et al., 2014)

<i>L. reuteri</i> NCIMB 30242	Randomised, double-blind, placebo-controlled, parallel, multi-centre	127 hypercholesterolemic volunteers	1 capsule d <sup>-1</sup> ( $5 \times 10^9$ CFU g <sup>-1</sup> ) or placebo for 9 weeks	LDL drop: 11.64% TC drop: 9.14%	(Jones et al., 2012)
<i>L. acidophilus</i> La5 and <i>B. lactis</i> Bb12	Randomised, double-blind controlled study	60 subjects with type 2 diabetes	300 g of probiotic yogurt /day containing $1.05 \times 10^6$ CFU g <sup>-1</sup> of <i>L. acidophilus</i> La5 and $1.19 \times 10^6$ CFU g <sup>-1</sup> of <i>B. lactis</i> Bb12 for 6 weeks	LDL: 4.54% decrease TC: 7.45% decrease	(Ejtahed et al., 2011)
<i>L. acidophilus</i> , <i>B. longum</i> & FOS	Randomised, single-blind, placebo - controlled & parallel	55 normo-cholesterolemic volunteers	3 capsules d <sup>-1</sup> ( $10^9$ CFU g <sup>-1</sup> of each probiotic and 10-15 mg of FOS for 2 months	No significant improvement in lipid profile	(Greany et al., 2008)
<i>L. fermentum</i>	Double-blind, placebo - controlled & parallel	44 healthy volunteers with $TC \geq 4$ mmol L <sup>-1</sup>	4 capsules d <sup>-1</sup> ( $2 \times 10^9$ CFU g <sup>-1</sup> <i>L. fermentum</i> ) for 10 weeks	No significant improvement	(Simons et al., 2006)
<i>L. acidophilus</i>	Double-blind, placebo-controlled cross-over	80 overweight volunteers (29 males and 51 females; 20-65 years old)	2 capsules containing $3 \times 10^{10}$ CFU g <sup>-1</sup> freeze-dried <i>L. acidophilus</i> , three times a day for 6 weeks	No significant improvement in lipid profile	(Lewis & Burmeister, 2005)
<i>L. acidophilus</i> 145 and <i>B. Longum</i> 913	Randomised, cross-over, placebo-controlled study	29 hypercholesterolemic female subjects, 19-56 years old	Daily intake of 300 g probiotic yogurt containing $10^6$ - $10^8$ CFU g <sup>-1</sup> of <i>L. acidophilus</i> 145 and $10^5$ CFU of <i>B. longum</i> 913 per gram yoghurt for 21 weeks	HDL increased by 0.3 mmol/L, LDL: HDL decreased from 3.24 to 2.48. (P < 0.05)	(Kieling et al., 2002)
<i>L. plantarum</i> 299v	Randomised, placebo-controlled, double-blind, parallel study	36 healthy volunteers, 35-45 years old, mean TC: $5.59 \pm 0.88$ mmol L <sup>-1</sup>	Daily intake of 400 mL of rose hip drink containing $5.0 \times 10^7$ CFU mL <sup>-1</sup> of the probiotic over 6 weeks	TC drop: 2.5% LDL drop: 7.9%	(Naruszewicz et al., 2002)
<i>Enterococcus faecium</i> and 2 strains of <i>S. thermophiles</i>	Randomised, placebo-controlled, double-blind, cross-over	32 subjects, 36-65 years old; mean TC: $248.47 \pm 26.75$ mg dL <sup>-1</sup> , mean LDL: $172.22 \pm 21.17$ mg dL <sup>-1</sup>	Daily intake of 200 g of Gaio containing $10^5$ - $10^9$ CFU mL <sup>-1</sup> of <i>E. faecium</i> & $5 \times 10^8$ CFU mL <sup>-1</sup> of <i>S. thermophiles</i> , for 16 weeks	TC drop: 5.3% (P = 0.004) LDL drop: 6.15% (P = 0.012)	(Bertolami et al., 1999)

While the hypocholesterolemic effect of probiotics has been well established in various studies, prebiotics have also gained increasing attention due to their role in the stimulation of the growth and activity of probiotics (Table 2.5). In addition, many animal and human studies have shown that consuming a mixture of probiotics and prebiotics in what is known as synbiotic foods can enhance the survival and activity of these organisms (see section 2.3).

Despite these reports on the cholesterol-lowering effects of prebiotics and probiotics in both animals and humans, some controversial findings are also reported that require further analysis (Table 2.5). Various factors may have contributed to these controversial findings. Although *in vivo* trials usually apply to real life models with true representations of pathoLogical systems, the validity of these trials may have been adversely affected by probiotic specifications, prebiotic characterizations, administration dosage, duration of treatment, experimental design, analytical accuracy of lipid measurement, clinical characteristic of subjects, inadequate sample sizes, and lack of suitable controls or placebo groups. Therefore, the potential hypocholesterolemic characteristics of probiotics and prebiotics still requires further research.

**Table 2.5:** *In vivo* studies on the controversial hypocholesterolemic effects of prebiotics

Prebiotics	Study design	Animals/Subjects	Dose, duration of the study	Effects	References
<b>Animal studies</b>					
GOS	Randomised, placebo- controlled and parallel study	Hypercholesterolemic female SD rats	3 groups, receiving 110 mg, 154 mg or 198.4 mg inulin per 250 g BW for 60 days	Significant reduction of serum TC, TG and LDL ( $P < 0.05$ )	(Hashmi et al., 2016)
Xylooligosaccharides (XOS)	Randomised, placebo controlled, Parallel study	40 x 6-week old male SD rats	60 g XOS kg <sup>-1</sup> BW for 35 days	TG drop: 34 % ( $P < 0.05$ )	(Hsu et al., 2004)
Inulin	Randomised, double-blind, placebo-controlled, parallel	40 male mice	10% of inulin daily 16 weeks	TC drop: 29.7% LDL drop: 25.9% ( $P < 0.01$ )	(Mortensen et al., 2002)
Resistant starch (RS)	Randomised, placebo-controlled and parallel study	16 male guniea pigs (BW:300-400 g)	10g RS per 100g BWday <sup>-1</sup> 4 weeks	LDL drop: 27.4%; TC drop: 28% ( $P < 0.01$ )	(Fernandez et al., 2000)
β-cyclodextrin	Randomised, placebo- controlled and parallel study	10 male Wistar rats (mean BW: 150 g)	25g kg <sup>-1</sup> BW per day for 21 days	TG drop:26%; TC drop: 35% ( $P < 0.05$ )	(Favier et al., 1995)
<b>Human Studies</b>					
GOS	Randomised, placebo-controlled, double-blind, and cross-over study	44 elderly volunteers (16 men, 28 women), 64-79 years old	5.5 g d <sup>-1</sup> , two × 10 weeks treatment period with 4-week washout period	No significant improvement in lipid profiles	(Vulevic et al., 2008)
Inulin	Randomised, placebo-controlled, double-blind study	8 subjects (4 men, 4 women), 3-32 yo	10g inulin day <sup>-1</sup> for 3 weeks	TG significantly reduced ( $P < 0.05$ )	(Letexier et al., 2003)
FOS	Randomised, placebo, double-blind & cross-over	10 diabetic subjects (6 men, 4 women)	20 g FOS day <sup>-1</sup> for 4 weeks	No improvement in lipid profile	(Luo et al., 2000)
Inulin	Randomised, cross-over, double-blind study	12 hyper-cholesterol emic male subjects	475 mL vanilla ice-cream /day plus 20g inulin, 3weeks	TG: significant decrease ( $P < 0.05$ )	(Causey et al., 2000)
Inulin	Randomised, cross -over, placebo-controlled study	12 normolipidemic young men	50 g cereals containing 18% inulin; 4- week treatment & 4-week wash out period	TC drop: 7.9%; TG drop: 21.2% ( $P < 0.05$ )	(Brighenti et al., 1999)

## 2.6 CHOLESTEROL REDUCTION MECHANISMS

### 2.6.1 Mechanisms of cholesterol reduction by probiotics

A number of cholesterol lowering mechanisms by *Lactobacillus* strains have been proposed (Corzo & Gilliland, 1999; Tanaka et al., 2000; Liong & Shah, 2005; Ramasamy et al., 2010). One such mechanism is the deconjugation of bile salts by probiotic bacteria which are able to convert glycin- or taurin- conjugated bile salts into amino acid residues and free bile acids (Corzo & Gilliland, 1999). Deconjugated bile acids are less efficiently reabsorbed than their conjugated counterparts, and are mostly excreted in the faeces. Also, free bile acids are less efficient in solubilisation and absorption of lipids in the gut. Therefore, deconjugation of bile salts could lead to a reduction in serum cholesterol either by increasing the demand for *de novo* synthesis of bile acids to replace those lost in faeces or by reducing cholesterol solubility and thereby blocking the absorption of cholesterol through the intestinal lumen (Walker & Gilliland, 1993). The excreted bile acids are replaced by new bile salts formed from cholesterol in the liver (Klaver & Van der Meer, 1993; Walker & Gilliland, 1993). Corzo and Gilliland (1999) have also reported that low pH would favour the secretion of BSH. Milk lactose is a potent energy source for *Lactobacilli* that produce lactic acid and SCFAs and thus reducing the pH. Therefore, fermented dairy products such as yogurt could enhance BSH production.

Walker & Gilliland (1993) reported that strains of *L. acidophilus* secrete BSH to deconjugate bile salts, whereas (Dambekodi & Gilliland, 1998) found no relationship to exist between the *in vitro* cholesterol reduction and the degree of bile salt deconjugation. These conflicting results lead to the possibility of the existence of other mechanisms which may be associated with cholesterol assimilation by probiotic bacteria during their growth in the media (Lye et al., 2010).

It has been reported that the membranes of growing cells of some probiotic strains can attach to cholesterol (Kimoto et al., 2002; Tok & Aslim, 2010), and some degree of attachment has

been observed even on dead cell membranes. Since the probiotics are regularly shed in the faeces, this effect results in mopping up of cholesterol from the GIT; however, this effect is highly strain-dependent (Pereira & Gibson, 2002).

Another proposed mechanism for cholesterol reduction is the conversion of cholesterol to 5 $\beta$ -coprostanol (5 $\beta$ -cholestan - 3 $\beta$ -ol) in the intestine (Bull et al., 2002). 5 $\beta$ -coprostanol is a 27 carbon stanol formed from the bio-hydrogenation of cholesterol in the intestine. It is less soluble than cholesterol and is associated with the solid phase in the gastrointestinal system, thus being directly excreted in the faeces (Bull et al., 2002). This eventually leads to a reduced concentration in the physiological cholesterol pool. The mechanism of cholesterol conversion into 5 $\beta$ -coprostanol by intestinal bacteria has been reported in different studies. Chiang et al. (2008) found that bacterial strains such as *Sterolibacterium denitrificans* were able to produce cholesterol dehydrogenase, responsible for catalyzing the transformation of cholesterol to cholest-4-en-3-one, an intermediate cofactor in the conversion of cholesterol to 5 $\beta$ -coprostanol. Lye et al. (2010) evaluated the mechanism of cholesterol conversion to 5 $\beta$ -coprostanol by strains of *Lactobacilli* including *L. acidophilus* ATCC 314, *L. bulgaricus* FTCC 0411 and *L. casei* ATCC 393 using fluorometric assays. The authors detected both intracellular and extracellular cholesterol reductase in all strains examined, indicating possible intracellular and extracellular conversion of cholesterol to 5 $\beta$ -coprostanol. This was evidenced by drop in cholesterol concentration in the fermentation medium accompanied with increased concentration of 5 $\beta$ -coprostanol. The authors concluded that these strains may have potential hypocholesterolemic properties and could be used in fermented dairy products as adjunct cultures.

Most of the reported cholesterol reduction mechanisms by probiotics are based on *in vitro* experiments, and only a few attempts have been made to evaluate this effect *in vivo*. The earlier *in vivo* trials aimed to verify the hypocholesterolemic effects of the probiotics, as well as

understand the exact cholesterol reduction mechanisms involved. For example, Liong et al. (2007) evaluated the possible mechanism of cholesterol reduction upon feeding twenty-four Crossbred hypercholesterolemic pigs with a synbiotic diet supplemented with freeze-dried *L. acidophilus* ATCC 4962 (1.00 g kg<sup>-1</sup>), FOS (1.25 g kg<sup>-1</sup>), mannitol (1.56 g kg<sup>-1</sup>) and inulin (2.20 g kg<sup>-1</sup>) for eight weeks. This feeding trial significantly lowered the plasma total cholesterol (P = 0.001), TG (P = 0.002), and LDL cholesterol (P = 0.045) of the pigs compared to the control group. The authors explained this effect to be due to the transport of cholesteryl esters via the interrelated pathways of lipid transporters (VLDL-C, LDL-C, and HDL-C), so that the concentration of cholesteryl esters is reduced in the LDL-C molecules and increased in the HDL-C molecules (Liong et al., 2007; Zhang et al., 2008). Thus HDL-C cholesterol plays a beneficial role in transporting the cholesterol to the liver for further hydrolysis.

### **2.6.2 Mechanisms of cholesterol reduction by prebiotics**

Prebiotics contribute to the regulation of cholesterol levels in both humans and animals. Although the mechanism is yet to be fully elucidated, two mechanisms have been proposed: 1) decreasing cholesterol absorption as a result of enhanced cholesterol excretion in the faeces, and 2) production of SCFAs upon selective fermentation by intestinal microflora (St-Onge et al., 2000; Kumar et al., 2012). SCFAs can lower the concentration of serum lipids through blocking the synthesis of hepatic cholesterol by inhibiting lipogenic enzymes such as acetyl-CoA carboxylase (EC 6.4.1.2), fatty acid synthase, malic enzyme (EC 1.1.1.40), ATP citrate lyase (EC 4.1.3.8), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Delzenne & Kok, 2001), or through redirecting the plasma cholesterol toward the liver (Xiong et al., 2004).

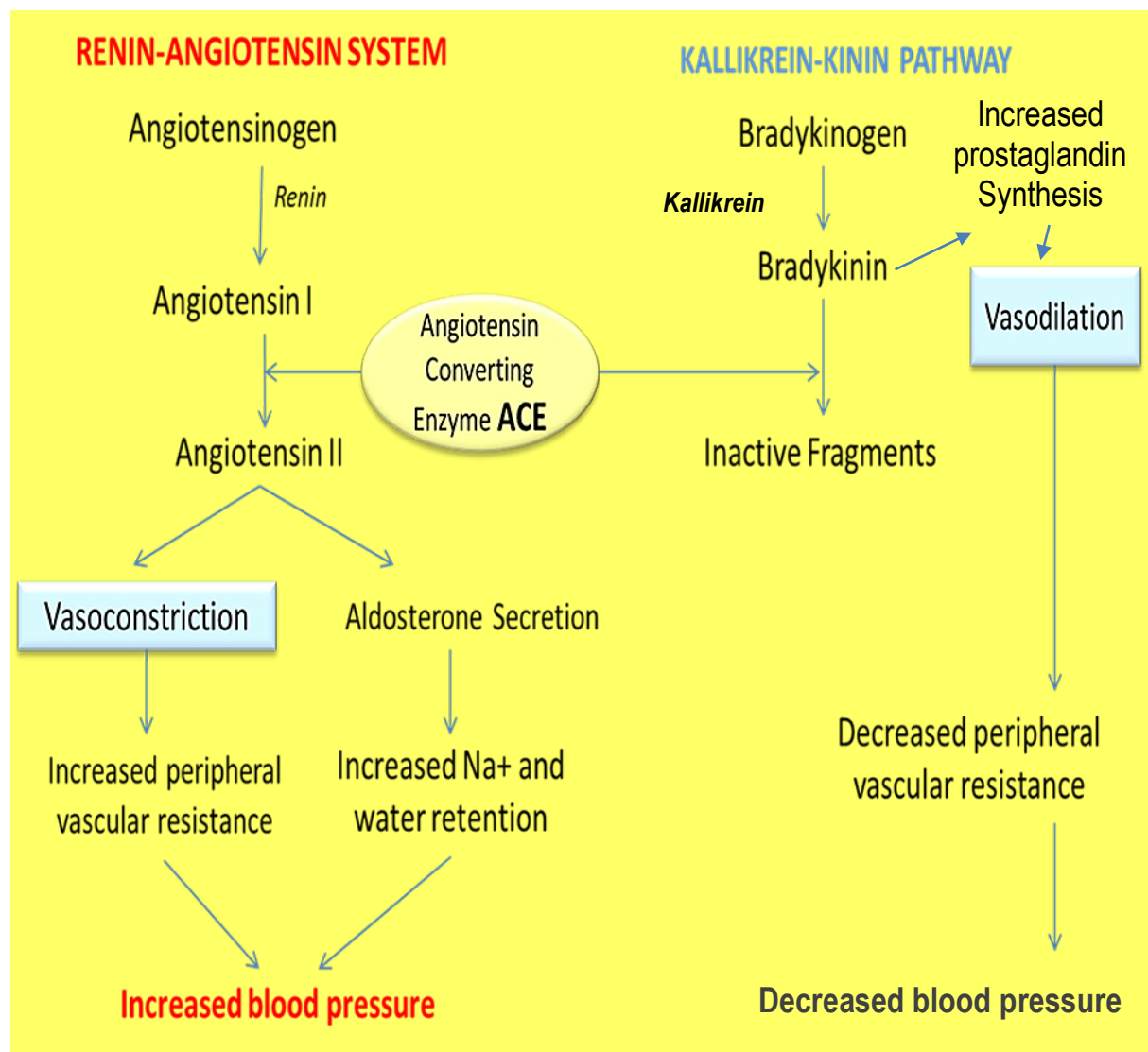
## **2.7 ANTI-HYPERTENSIVE MECHANISMS**

### **2.7.1 Anti-hypertensive effects of probiotics**

Many studies have reported the links between elevated blood pressure and a number of biochemical pathways including rennin-angiotensin system (RAS) which involves angiotensin-converting enzyme (ACE, peptidyl dipeptide hydrolase) that plays a crucial role in the regulation of blood pressure, fluid and electrolyte balance.

The angiotensin-converting enzyme converts the inactive decapeptide angiotensin I by cleaving a dipeptide from the C-terminus into angiotensin II which is a potent vasoconstrictor. Angiotensin II is also involved in the release of a sodium-retaining steroid, aldosterone, from the adrenal cortex, which has a tendency to increase the blood pressure (Johnston, 1992). ACE also catalyses the degradation of bradykinin, a vasodilatory nonapeptide, via the Kallikrein-Kinin pathway (Figure 2.1). Inhibition of ACE is thus considered a useful therapeutic approach in the treatment of hypertension. ACE inhibition may also influence other regulatory systems involved in immune defence, and nervous system activity (Meisel, 1998).





**Figure 2.1** Role of angiotensin-I converting enzymes in blood pressure regulation (Li et al., 2004).

The  $\beta$ -casein molecule in milk contains amino acid sequences with ACE-inhibiting effects that are inactive unless released by the proteolytic action of digestive and microbial enzymes (Korhonen, 2009). Therefore, milk fermentation is considered an effective way to release these bioactive peptides. ACE inhibitory peptides are found in a variety of fermented milk products including yogurt, fermented milk beverages and matured cheeses. In addition to starter cultures, the probiotic bacteria have also been demonstrated to release different bioactive peptides during milk fermentation (Korhonen, 2009). Probiotics possess a proteolytic system that degrades casein to release bioactive peptides, including ACE inhibitory peptides. Korhonen

(2009) has demonstrated the ability of *L. helveticus* to release ACE inhibitory tripeptides isoleucyl-prolyl-proline (Ile-Pro-Pro) and valyl-prolyl-proline (Val-Pro-Pro) from  $\beta$ -casein. Various *in vitro* and *in vivo* studies have documented the beneficial effects of consuming probiotic dairy foods on hypertension. The anti-hypertensive properties of probiotics has also been confirmed by Ong and Shah (2008), who examined the release of ACE inhibitory peptides in cheddar cheese containing *L. Saz 4casei* and *L. acidophilus* that showed increased proteolysis and higher ACE inhibitory activity after 24 weeks of ripening, compared to those made without any probiotic adjunct cultures. It can therefore be postulated that dairy foods containing probiotics provide a natural means of controlling hypertension in hypertensive populations. *In vivo* studies on hypotensive effects of probiotic strains is summarized in Table 2.6.

**Table 2.6:** *In vivo* studies on the hypotensive effects of probiotic strains

<b>Probiotic</b>	<b>Study design</b>	<b>Subjects</b>	<b>Dose, duration of the study</b>	<b>Effects</b>	<b>Reference</b>
<i>L. helveticus</i>	Randomised, placebo-controlled study	39 mild hypertensive subjects, 30-62 years old	150 mL fermented milk per day for 21 weeks	<b>SBP:</b> 6.7 mm Hg drop <b>DBP:</b> 3.6 mm Hg drop	(Seppo et al., 2003)
<i>L. acidophilus</i> and <i>S. thermophilus</i>	Randomised, double-blind, placebo and compliance-controlled parallel study	70 healthy volunteers, 18-55 years old	450 mL of probiotic yogurt per day for 8 weeks	<b>SBP:</b> 4.4 mm Hg drop <b>DBP:</b> 3.4 mm Hg drop	(Agerholm-Larsen et al., 2000)
<i>L. helveticus</i> CM4	Randomised, double-blind, placebo – controlled study	40 subjects with high blood pressure and 40 subjects with mild hypertension	6 tablets (12 g) per day containing fermented milk powder with <i>L. helveticus</i> CM4; for 4 weeks	<b>SBP:</b> 5.0 mm Hg drop <b>DBP:</b> 11.2 mm Hg drop	(Aihara et al., 2005)
<i>L. helveticus</i>	Randomised, double-blind, placebo-controlled study	46 borderline hypertensive subjects, 23-59 years old	160 g sour milk drink day <sup>-1</sup> for 4 weeks	<b>SBP:</b> 5.2 mm Hg drop <b>DBP:</b> no significant difference	(Mizushima et al., 2004)
<i>L. helveticus</i> and <i>S. cerevisiae</i>	Randomised, placebo-controlled study	30 elderly hypertensive subjects	95 ml of fermented sour milk per day for 8 weeks	<b>SBP:</b> 14.1 mm Hg drop <b>DBP:</b> 6.9 mm Hg drop	(Hata et al., 1996)

### **2.7.2 Anti-hypertensive effects of prebiotics**

One of the possible antihypertensive mechanisms of prebiotics could be explained through their ability to lower the blood lipids and be attributed to their fermentation by selective colonic microflora to produce SCFA such as acetate, propionate and lactate (Ferrier et al., 2002). Insoluble prebiotics are resistant to digestion in the small intestine and reach the colon to get fermented by lactic acid and probiotic bacteria to produce lactate that is further fermented to acetate and propionate (Levrat-Verny et al., 2000). It has been reported that propionate could delay fatty acids and cholesterol synthesis, whereas lactate usually slows down the production of TG (Watson & Preedy, 2010). As mentioned earlier, the prebiotics are able to inhibit the intestinal cholesterol and bile absorption (Levrat-Verny et al., 2000; Gallaher et al., 2002) that regulates the LDL cholesterol receptors and reduces the stiffness of arteries, thus potentially reducing blood pressure (Ferrier et al., 2002).

An excess body weight has been associated with overactivity of the sympathetic nervous system, peripheral vasoconstriction, increasing renal tubular sodium reabsorption and raising arterial pressure (Lairon et al., 2005). Therefore, dietary management of obesity with prebiotic consumption could also help reduce the development of hypertension (Lairon et al., 2005; Rahmouni et al., 2005). Cani et al. (2005) have shown that an addition of 10% inulin-type fructans into the high-fat rat diet for thirty-five days could regulate body weight and protect against fat mass development via the promotion of endogenous Glucagon-like Peptide-1 (GLP-1) in the gut. GLP-1 plays an important role in glucose homeostasis, energy metabolism, gastrointestinal motility and appetite. Furthermore, Cani et al. (2006) confirmed that the addition of 16 g per day of FOS to the diet of ten human volunteers for two weeks significantly ( $P \leq 0.05$ ) reduced the total energy intake by 5% and increased satiety following breakfast and dinner meals. Another proposed antihypertensive mechanism of prebiotics includes the

reduction of insulin resistance (Saad et al., 2004). Insulin resistance is a metabolic disorder that has been shown to be related to the development of hypertension via reduced insulin-induced vasodilation and decreased metabolism in adipocytes and skeletal muscles. Insoluble prebiotics are able to improve postprandial glucose response, decrease insulin secretion and increase insulin sensitivity and therefore, reduce the risk of HT development (Robertson et al., 2003; Saad et al., 2004). In addition, it has been shown that prebiotics could reduce the blood pressure by improving the absorption of calcium in the GIT (Streppel et al., 2005). Allender et al. (1996) conducted a meta-analysis of randomised clinical trials investigating the impact of dietary calcium on blood pressure and found that increasing calcium intake by 1 g per day in hypertensive subjects was associated with a statistically significant decrease in SBP of 1-2 mm Hg. Scholz-Ahrens et al. (2001) have shown that  $\text{Ca}^{+}$  absorption in the human GIT is highly dependent on its bioavailability and the level of absorption in the intestinal lumen. Prebiotics such as inulin have been reported to increase the absorption of  $\text{Ca}^{+}$  via binding to calcium in the upper GIT (Roberfroid, 2000; Scholz-Ahrens et al., 2007), that upon reaching the colon is released and reacts with the  $\text{H}^{+}$  of SCFA in distal colon regions, thus increasing the concentration of free  $\text{Ca}^{+}$  available for absorption (Scholz-Ahrens et al., 2007).

**Table 2.7:** *In vivo* studies on the hypotensive effects of prebiotics

<b>Prebiotic</b>	<b>Study design</b>	<b>Subjects</b>	<b>Dose, duration of the study</b>	<b>Effects</b>	<b>Reference</b>
Water-soluble fibre extracted from oat bran	Randomised, double-blind, placebo-controlled study	110, 30-65 years old normotensive subjects,	8 g per day of fibre for 12 weeks	<b>SBP:</b> 2.0 mm Hg drop <b>DBP:</b> 1.0 mm Hg drop	(He et al., 2004)
Psyllium fibre mixed with soy protein isolate	Randomised, double-blind, parallel study	Non-smoking men or women > 20 years on antihypertensive drug for > 6 months with SBP of 130-160 mm Hg	12 g fibre per day for 8 weeks	<b>SBP:</b> 5.9 mm Hg drop <b>DBP:</b> not significant	(Burke et al., 2001)
Dietary fibre	Randomised, double-blind, parallel, placebo controlled	63 hypertensive 18-70 years old subjects with a min DBP > 90 mm Hg, ,	7 g dietary fibre per day for 12 weeks	<b>SBP:</b> not significant <b>DBP:</b> 5.0 mm Hg drop	(Eliasson et al., 1992)
$\beta$ -glucan from whole oats	Randomised, parallel, pilot trial	18 hypertensives 27-59 years old subjects with SBP 130-160 mm Hg and DBP 85-100 mm Hg,	5.52 g $\beta$ -glucan per day for 6 weeks	<b>SBP:</b> 7.5 mm Hg drop <b>DBP:</b> 5.5 mm Hg drop	(Keenan et al., 2002)
Lupin kernel flour	Randomised parallel study	74 mild hypertensive 20-70 years old subjects with SBP <150 mm Hg and DBP <95 mm Hg,	4 x 40g of bread day <sup>-1</sup> , bread containing 9.5% (w/w) of fibre for 16 weeks	<b>SBP:</b> 3.0 mm Hg drop <b>DBP:</b> not significant	(Lee et al., 2009)

## 2.8 CONTROVERSIAL STUDIES

Although the majority of *in vitro* and *in vivo* studies report that the addition of prebiotics into daily foods could reduce cholesterol levels and improve blood pressure; however, controversies are raised where a number of studies have reported otherwise (Tables 2.6 and 2.7). Future studies investigating the effects of prebiotics on serum cholesterol or blood pressure should consider the choice of study subjects, duration of supplementation and sample size. Past studies investigating the effects of non-digestible oligosaccharides (NDOs) such as inulin and FOS consumption on HT and cholesterol reduction remains relatively controversial and requires future investigation. Therefore, optimisation of the formulation of a synbiotic food product is being attempted for the maximum *in vitro* hypotensive and hypocholesterolemic effects using different types of probiotic bacteria and prebiotics. This optimised formulation will be further explored in the human trial.

## 2.9 DOSE-RESPONSE EFFECTS OF PREBIOTICS AND PROBIOTICS

Despite many health benefits claimed about prebiotics and probiotics, an accurate administration dose is yet to be established. There is a lack of dose-response studies to determine the ‘minimal effective dosage’ of prebiotics and probiotics needed to reduce blood pressure and cholesterol levels. The concentration of prebiotics and probiotics in food products varies extremely and there is currently no standard for probiotic dosage to deliver a cholesterol-lowering effect (FSANZ, 2013). Various studies have confirmed that effective administration dosage of probiotics is strain-dependant and highly affected by the clinical history of the subjects, such as their serum lipid profiles (Lubbadeh et al., 1999; Ha et al., 2006).

Several studies have been conducted to determine the use of various formulations of synbiotics for cholesterol-lowering effects. Liong and Shah (2006), in an attempt to optimise *in vitro* cholesterol reduction, achieved the maximum result by combining  $10^9$  CFU g<sup>-1</sup> of

freeze-dried *L. casei* ASCC 292, with 4.95% (w/v) FOS and 6.64% (w/v) maltodextrin. This optimised formulation was further explored in an animal model where twenty-four hypercholesterolemic male Wistar rats were fed daily with a synbiotic diet for six weeks and showed a significantly ( $P < 0.05$ ) lower total cholesterol levels and TG (16.7% and 27.1%, respectively) compared to the control group. However, rats fed the same diet with either FOS or maltodextrin showed no significant reduction in their lipid profile, which confirms the effectiveness of synbiotic foods. Similarly, Zhang et al. (2007) observed that *L. plantarum* LS12 in the presence of GOS and mannitol could reduce 76% more cholesterol compared to the control that contained only prebiotic-supplemented medium.

Thus, more studies are needed, not only to determine the effective dosage of synbiotics to deliver the maximum hypocholesterolemic effects, but also to evaluate the interaction between probiotics and prebiotics that enhances their cholesterol-lowering properties.

## **2.10 SAFETY OF PREBIOTICS AND PROBIOTICS**

Most strains of probiotics such as *L. rhamnosus*, *L. casei*, *L. paracasei*, and *L. plantarum*, *B. longum* and *B. bifidum* are recognised as the normal GIT microflora and are generally safe for consumption (Olano et al., 2001; Chazan et al., 2008). However, their potential pathogenicity and impact on gut microflora are assessed by WHO and by other national or international agencies responsible for food safety or public health (FSANZ, 2013). Some of the adverse effects that might be induced by probiotics include impaired immune system, deleterious metabolic activities, genetic interactions between probiotics and the GIT microbes and occurrence of antibiotic resistance (Ooi & Liong, 2010).

It has been suggested that upon probiotic ingestion, the microorganism load passing through the small intestine is increased, resulting in gastrointestinal disturbances, such as intestinal inflammation. Tan et al. (2007) have hypothesised that BHS production by probiotic strains



may increase the accumulation of deconjugated bile salts, which could be subsequently transformed into detrimental bile acids by intestinal microflora that may increase the risk of colorectal cancer.

The genetic interactions between ingested probiotics and the intestinal microbes have been another focus of interest. Shimizu et al. (2005) tried to ascertain whether any genetic exchange, e.g. transduction, conjugation or transformation occurred between probiotics and the indigenous GIT microflora. It is claimed that the transformation of intestinal microflora by microinjected DNA from live cells may be enhanced upon ingestion of probiotic bacteria and act as a pool for the transmission of antibiotic-resistance genes from beneficial bacteria to harmful pathogens that may lead to the evolution of antibiotic-resistant pathogens. D'Aimmo and Modesto (2007) studied thirty-four strains of *Lactobacillus* and *Bifidobacterium* and twenty-one strains of lactic acid bacteria (including *Streptococcus thermophilus*) isolated from dairy products for their antibiotic resistance and found that all strains were resistant to aztreonam, cycloserin, kanamycin, and nalidixic acid. Similarly, Hummel et al. (2007) evaluated antibiotic resistant genes in forty-five strains of probiotics from *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* genera using polymerase chain reaction (PCR) and found that 78 % of the probiotic strains examined were resistant to gentamicin, streptomycin and ciprofloxacin.

Prebiotics such as inulin and FOS are present in our daily dietary intake and their daily intake has been estimated at up to 8-10 g in the population of Australia and New Zealand (FSANZ, 2008). The safety of inulin and FOS for food application was evaluated by many legal authorities worldwide, and *in vivo* experimental evidence has not demonstrated any toxic effects (FSANZ, 2008). However, prebiotics at very high doses might increase incidences of bloating, flatulence and high osmotic pressure, which lead to gastrointestinal discomfort. The effects might vary widely between individuals and depend on the type of food in which the

prebiotics are incorporated (Ooi & Liong, 2010). Some studies have found that dietary FOS increased the intestinal translocation of the invasive pathogen *Salmonella enteritidis* to the extra-intestinal organs in new-born mice (Ten Bruggencate et al., 2004; Ten Bruggencate et al., 2005). Moreover, FOS increased infection-induced growth impairment, diarrhoea, gut inflammation and permeability of the large intestine, increase cytotoxicity of the intestinal contents, mucin excretion, and intestinal permeability in rats (Ten Bruggencate et al., 2004). As per results of various clinical studies, probiotics and prebiotics are considered generally safe for consumption due to their low ability of triggering adverse health effects (Roberfroid, 2007). Currently, there is no standard analysis or assay suggested for safety assessment on probiotics and prebiotic. However, each probiotic and prebiotic should be evaluated for safety, so that these strains can be isolated for specific purposes at specific dosages to prevent potential adverse reactions (Ooi & Liong, 2010).

## **2.11 POMEGRANATE HEALTH BENEFITS**

Health-conscious consumers across the world are becoming more interested in foods with health-promoting functions as they gain more awareness of the nexus between food and health (Paseephol & Sherkat, 2009). Phenolic compounds produce their beneficial effects by scavenging free radicals. There has been an increasing interest in determining dietary sources of phenolic compounds in red fruits and their juices due to their high antioxidant contents (Fischer et al., 2011; Akhavan et al., 2015).

Pomegranate juice (PJ) has become more popular since the latter part of the 20<sup>th</sup> century due to a better understanding of its high antioxidant content compared to other fruit juices and beverages (Howell & D'Souza, 2013). Seeram et al. (2008) evaluated the popular polyphenol-rich beverages available in the United States market for their total phenolic compounds (TPC), antioxidant activity (AA) and function on cholesterol reduction. The selected beverages

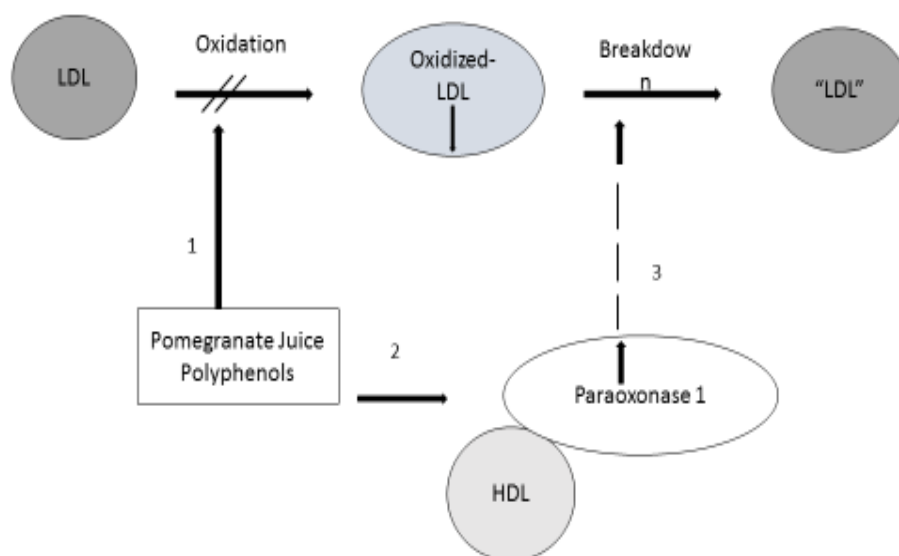
included three different brands of apple juice, black cherry juice, blueberry juice, cranberry juice, grape juice, orange juice, red wine, iced tea beverages, and PJ. The ranking of the beverages on the basis of the average amounts of TPC was: PJ > red wine > grape juice > blueberry juice > black cherry and cranberry juices > orange juice, iced tea beverages and apple juice. Both antioxidant activity and antioxidant functionality of the beverages were highly correlated with their TPC levels. -The PJ showed 20% more AA than all the beverages tested. Pomegranate juice is a very popular beverage in different parts of the world, especially in the Middle Eastern countries where it is produced in large quantities. Consequently, a large number of industries have been developed in these countries for the extraction, processing and packaging of PJ, as well as pharmaceutical companies for isolating health promoting compounds from different parts of the pomegranate (Lansky et al., 1998; Seeram et al., 2008).

Tezcan et al. (2009) suggested that PJ increases HDL activity associated with paraoxonase 1 (PON1) and decreases LDL's susceptibility to aggregation and oxidation (Figure 2.2). PON1 is a liver enzyme which plays an important role in preventing the oxidation of LDL and development of atherosclerosis. The serum concentration of PON1 is influenced by inflammatory changes and the levels of serum oxidised-LDL (Bergmeier et al., 2004; Richter, 2010).

Pomegranate juice can inhibit LDL oxidation in three ways: 1) the polyphenols in PJ inhibit copper ion-induced LDL oxidation, and thus reduce the oxidized LDL content, 2) These polyphenols also increase the activity of serum HDL-associated paraoxonase 1 (PON1), and 3) PON1 can in turn hydrolyse lipid peroxides in oxidized LDL and convert them to a less atherogenic "LDL", leading to a further reduction in oxidized LDL content (Rosenblat & Aviram, 2006) (Figure 2.2).

Methanol extracts of pomegranate fruit, especially the peel, exhibit broad-spectrum antibacterial activity, which can vary depending on the pomegranate variety tested (Howell & D'Souza,

2013). Methanol extracts of pomegranate are high in hydrolysable tannins (punicalins and punicalagins), ellagic acid, a component of ellagitannins, and gallic acid, a component of galloateannins. Furthermore, mass spectrometry data shows that pomegranate contains oligomeric ellagitannin with a DP of up to 5 core glucose units. These molecules may be the most potent antibacterial compounds in pomegranate as they exhibit strong activity against some species of bacteria such as *Bacillus subtilis*, *Shigella*, *Salmonella*, and *Staphylococcus aureus*, which justifies its use as a bio-preservative in foods (Aviram, 2002; Naz et al., 2007; Al-Zoreky, 2009; Howell & D'Souza, 2013). PJ consumption also helps keep the prostate specific antigen (PSA) levels stable in men and even slows its rise by extending PSA doubling time (Pantuck et al., 2006). Clinical studies provide evidence of antiproliferative properties of phytochemical-rich foods such as PJ. The ellagitannins in PJ have demonstrated anti-tumor activity *in vitro* and *in vivo* in human PCA cells through downregulation of NF- $\kappa$ B, cyclin dependent kinases 2/4/6 and Bcl-2 and upregulation of p21/WAF1 (Malik & Mukhtar, 2006; Paller et al., 2013). Pomegranate juice is also helpful against CVD development (Aviram et al., 2008), some types of cancer such as prostate and colon cancers (Adams et al., 2006; Adhami & Mukhtar, 2007) and reduces systolic blood pressure (Aviram & Dornfeld, 2001).



**Figure 2.2** Mechanism of LDL oxidation inhibition by pomegranate juice polyphenols (Rosenblat & Aviram, 2006)

## 2.12 NATURAL ANTIOXIDANTS AND HEALTH BENEFITS

The health benefits attributed to the consumption of fruits, vegetables and cereals, at least in part, are related to their antioxidant activity. Many constituents of these dietary components may contribute to their protective properties, including vitamins C and E, selenium and other trace minerals and micronutrients, carotenoids, phytoestrogens, glucosinolates and indoles, dithiolthiones, isothiocyanates, protease inhibitors, fibre and folic acid. These compounds may act independently or in combination as anti-cancer or cardio-protective agents by a variety of mechanisms, including radical-scavenging activity (Rice-Evans et al., 1997; Rosenblat & Aviram, 2006; Howell & D'Souza, 2013).

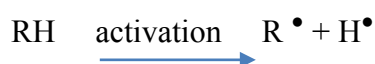
For a compound to be defined as an antioxidant it must satisfy two basic conditions:

1. When present in low concentration relative to the substrate to be oxidized, it can delay or prevent oxidative reaction or free radical-mediated oxidation.

2. The resulting radical formed after scavenging must be stable in order to interrupt the oxidation chain reaction (Rosenblat & Aviram, 2006).

The oxidative reaction involves a free-radical chain process that can be initiated by the action of external agents such as heat, light or ionizing radiation (Ingold, 1996) involving the following steps:

1) Initiation



2) Propagation



3) Chain-breaking termination (inhibition)



Where,

RH: the organic substrate

ROO<sup>•</sup>: the corresponding peroxy radical

ROOH: the hydroperoxide

AH: the antioxidant

A<sup>•</sup>: the stable, comparatively un-reactive radical.

Phytochemicals are the secondary metabolites produced by plants as a defence mechanism against environmental threats including harmful ultraviolet (UV) radiation, pathogens, and herbivorous predators. Plant polyphenols are a major group of phytochemicals and an important class of antioxidants. These compounds are virtually widespread in all plant foods, often at high levels (Pietta, 2000). Phenolic compounds are excellent oxygen radical scavengers because the electron reduction potential of the phenolic radical is lower than that of oxygen

radicals. Therefore, phenolic compounds can scavenge reactive oxygen intermediates, thus preventing further oxidative reactions (Ainsworth & Gillespie, 2007).

### **2.13 POMEGRANATE POLYPHENOLS**

Different types of phytochemicals have been identified in various parts of the pomegranate plant, including fruits and seeds. However, the major class of pomegranate phytochemicals are the polyphenols, which predominately are found in the fruit (Seeram et al., 2006). The edible portion of pomegranate fruit represents on average 52% (w/w) of total fruit, comprising 78% juice and 22% seeds. The fresh PJ contains 85.4% water (w/v), 10.6% (w/v) total sugars, 1.4% (w/v) pectin, 0.1% citric acid, 0.7% ascorbic acid, 19.6 mg free amino nitrogen and 50 mg ash. The seeds are rich in protein, crude fibre and ash representing 13.2, 35.3, and 2.0 % (w/w), respectively. Pomegranate seeds also contain 6.0% (w/w) pectin and 4.7% (w/w) carbohydrate. The iron, copper, sodium and magnesium contents of the juice are relatively lower than those of seeds, except potassium (Arjmand, 2011).

Pomegranate grows as a shrub or small tree reaching 4-10 m. The fruit size can vary from 6-12 cm in diameter and has a tough, leathery skin; its leaves are simple and the flowers are bisexual, with radial symmetry. The fruit is classified as a berry consisting of many closely packed red grains (arils), and irregular segments separated by non-edible white piths and thin membranes. Each aril contains a seed surrounded by edible juicy pulp (Eccles, 2009).

Rosenblat and Aviram (2006) found different types and levels of polyphenols including flavonoids and hydrolysable tannins (HT) in pomegranate fruit. Flavonoids including flavonols and anthocyanins are mainly found in the peel and arils. Hydrolysable tannins are found in the peel, membrane and pith and consisted of gallotannins, ellagitannins (ET), condensed tannins, mainly found in the peel and juice and, organic acids including gallic acid, chlorogenic acid and citric acid, mainly found in the juice.

Gil et al. (2000) postulated that HTs are the predominant polyphenols in PJ responsible for approximately 92% of its antioxidant activity. HTs are classified into two categories: gallotannins (1, 2, 4, 6-tetra-O-galloyl-D-glucose and 1, 2, 3, 4, 6-penta-O-galloyl-D-glucose) and ellagitannins (ellagic acid esters of D-glucose with one or more galloyl substitutions). They are susceptible to enzymatic and non-enzymatic hydrolysis and could be further classified, depending on the products of hydrolysis (Table 2.8). The predominant and unique gallagyl ester of pomegranate HTs is punicalagin, which is responsible for about half of the antioxidant activity of the juice (Seeram et al., 2006). During industrial juice extraction by hydrostatic pressing, the water soluble peel punicalagins are dissolved in the juice, and thus contributing to the outstanding antioxidant activity observed in commercial pomegranate juices (Gil et al., 2000).

#### **2.14 EVALUATION OF TOTAL PHENOLIC COMPOUNDS (TPC) AND ANTIOXIDANT ACTIVITY (AA) IN POMEGRANATE JUICE**

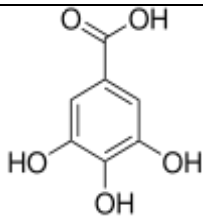
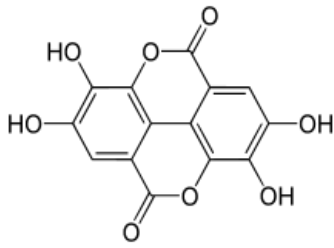
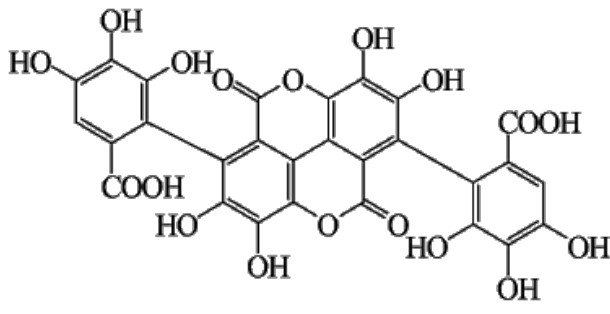
The available methods for quantification of TPC in food products or biological samples are based on the reaction of phenolic compounds with a colorimetric reagent, which allows measurement in the visible light portion of the spectrum. However, Martin et al. (2009) postulated that due to the remarkable complexity and heterogeneity of pomegranate polyphenols, the use of gallic acid as the standard leads to a significant underestimation of the TPC level.

The Folin-Ciocalteu (F-C) assay has been proposed by Singleton and Rossi (1965) as a standard method for use in routine quality control and measurement of phenolic compounds in food products and dietary supplements (Ainsworth & Gillespie, 2007). Gallic acid, a monomeric trihydroxylated molecule, is generally recognised as the standard of choice for



polyphenol measurement in the F-C assay, where the results are reported as milligram gallic acid equivalents (GAE) (Martin et al., 2009).

**Table 2.8** Composition and chemical structure of pomegranate hydrolysable tannins

Molecule	Composition	Chemical structure
Gallic acid	benzene ring with 1 carboxyl group in position 1 & 3  hydroxyl groups in positions 3,4, and 5	
Ellagic acid	gallic acid + gallic acid	
Gallagic acid	ellagic acid + ellagic acid	

(Martin et al., 2009)

It is possible to measure individual antioxidant components in a sample, but this is both time-consuming and expensive. In addition, since there seems to be an interaction between antioxidants during oxidative stress, examining one in isolation from the rest may not accurately reflect their combined action. Interest has therefore been focused on the measurement of total AA in biological samples. Such methods regard antioxidant activity as an overall characteristic of the product, regardless of the contribution of the individual compounds. In many cases, a relatively simple analysis of the foodstuff is sufficient, since the methods are based on measuring the inhibition of certain reactions in the presence of antioxidants (Cano et al., 1998). The most frequently used methods involve the generation of free radical type compounds, where the presence of antioxidants causes the free radicals to disappear (Rice-Evans & Miller, 1994).

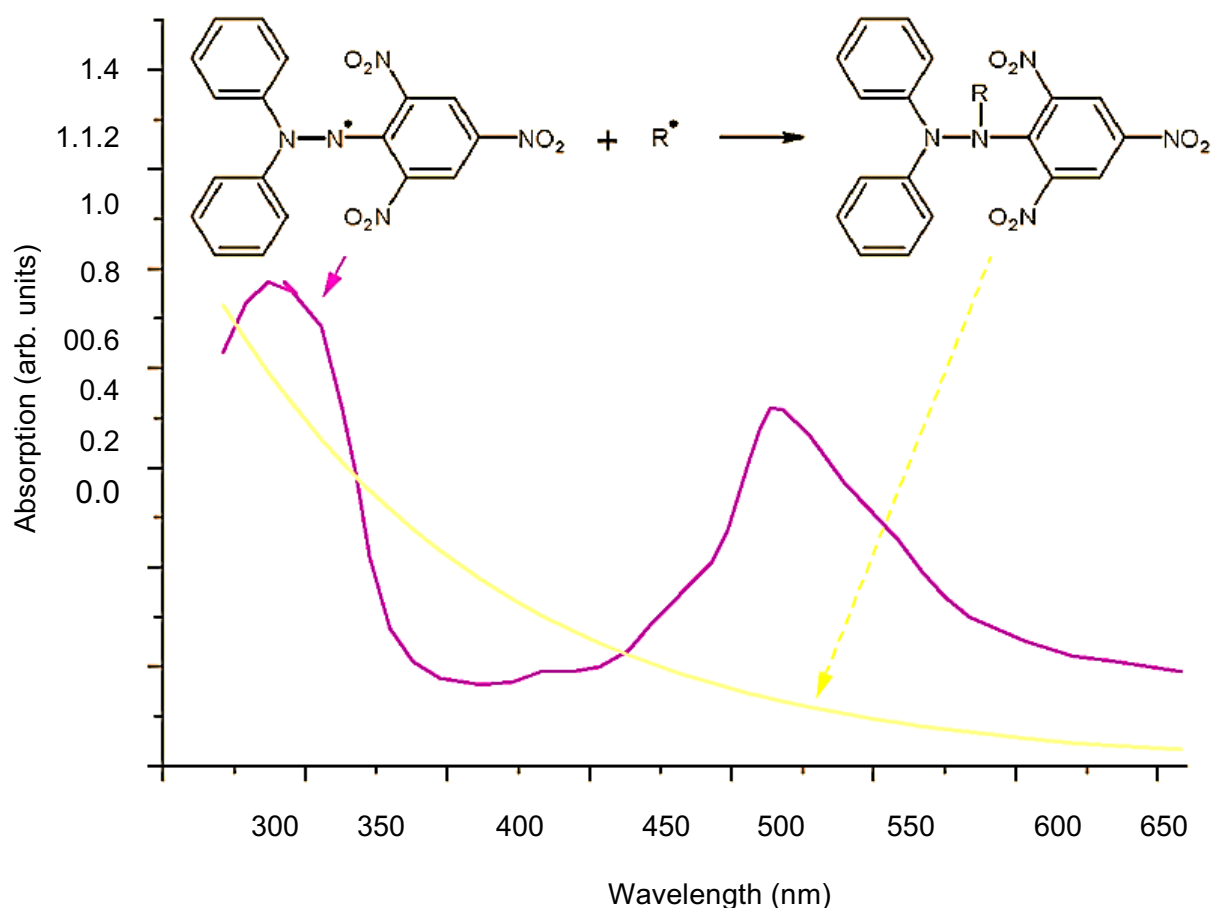
This method is based on the evaluation of the free radical scavenging capacity of the samples, the antioxidant activity of the samples is expressed as:

$$\text{Inhibition percentage \%} = [1 - (\text{absorbance of sample} / \text{absorbance of control})] \times 100\%$$

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay is one of the most popular and frequently employed methods to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate the antioxidant capacity of foods. The DPPH radical is a long-lasting organic nitrogen radical with a deep purple colour. It is commercially available and does not have to be generated before assay as in other scavenging assays. When a solution of DPPH radical is mixed with an antioxidant/reducing compound, its colour turns from purple to yellow of the corresponding hydrazine (Figure 2.3). Representing the DPPH radical by  $Z^{\bullet}$  and the donor molecule by  $AH^{\bullet}$ , the primary reaction is:  $Z^{\bullet} + AH = ZH + A^{\bullet}$

Where ZH is the reduced form and  $A^{\bullet}$  is free radical produced in the first step. This reaction is therefore intended to provide a link with the reactions taking place in an oxidising system, such as the autoxidation of a lipid or other unsaturated substance; the DPPH molecule  $Z^{\bullet}$  is thus

intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance AH. While DPPH can accept an electron or hydrogen radical to become a stable, diamagnetic molecule, it can be oxidized only with difficulty, and then irreversibly. DPPH shows a strong absorption band at 517 nm due to its odd electron and the solution appears a deep violet colour, the absorption then vanishes as the electron pairs off. The resulting decolourization is stoichiometric with respect to the number of electrons taken up. DPPH assay is a rapid, simple, inexpensive and widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate the antioxidant activity of foods. It can also be used to quantify antioxidants in complex biological systems for solid or liquid samples. This method is easy and is used to measure the overall antioxidant capacity and the free radical scavenging activity of fruit and vegetable juices. Moreover, this assay has been successfully utilised for investigating the antioxidant properties of fruit and vegetable juices, wheat grain and bran, vegetables, conjugated linoleic acids, herbs, edible seed oils, and flours in several different solvent systems including ethanol, aqueous acetone, methanol, aqueous alcohol and benzene, and for these reasons the DPPH method will be used as a method of choice in this study.



**Figure 2.3** Change in absorption spectrum ( from violet to yellow) upon reaction of DPPH radical with an antioxidant compound  
(Abuin et al, 2002, p.147)

## 2.15 FUNCTIONAL FOODS CONTAINING POMEGRANATE

The concept of “functional food” involves the use of bioactive ingredients or the presence of natural healthy bioactive molecules in foods (Coisson et al., 2005). A food can be regarded as “functional” if it satisfactorily demonstrates it has a beneficial effect on one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being or reduced risk of disease. Pomegranate products which contain health-promoting bioactive polyphenols could be considered as “functional” ingredients for their anti-free radical activities (Tomas-Barberan et al., 2006; Arjmand, 2011).

### 2.15.1 Yogurt as a carrier of probiotics and prebiotics

Since the renewed interest in probiotics, different types of products have been proposed as carriers for probiotics by which consumers can ingest large numbers of their cells for therapeutic effect (Lourens & Viljoen, 2001). Yogurt is a typical fermented dairy product consumed all around the world and is recognised as a ‘healthy snack’ with desirable health benefits. In Australia, yogurt consumption has increased steadily from 5.6 kg per capita in 2001 to 7.1 kg in 2015/2016 (Dairy Australia, 2016). Yogurt is prepared by allowing milk to ferment by starter cultures (*Streptococcus. thermophilus* and *Lactobacillus. bulgaricus*). Probiotic bacteria, which are incorporated in dairy foods, grow slowly during product manufacturing and include *Lactobacillus. acidophilus*, *Bifidobacterium* spp., *L. casei*, *L. rhamnosus*, and *Propionibacteria* (Shah, 2000; Paseephol & Sherkat, 2009).

It has been shown that the viability of probiotics over the product shelf-life is dependent on the type of yogurt. Birollo et al. (2000) observed that in whole set-yogurt the level of *Streptococci* dropped approximately  $1\text{Log}_{10}\text{ CFUg}^{-1}$  at 6 °C in 60 days shelf-life, whereas in skimmed yogurt and under similar conditions they remained viable and even multiplied. This indicates that high milk fat content may have an inhibitory effect for probiotic cultures, particularly on *B. bifidum*. In addition, survival of *L. acidophilus* is affected by yogurt post storage pH, therefore, any added ingredients such as parts of fruit that may lower the pH may further reduce the viability of *L. acidophilus* over the shelf-life (Ranadheera et al., 2010). On the other hand, the addition of substances such as whey protein into yogurt may enhance the probiotic’s viability due to increasing the buffering capacity of yogurt (Amatayakul et al., 2006)

Incubation time of the product also affects the viability of probiotics in yogurt. The yogurt starter cultures are anaerobic and do not scavenge oxygen. Instead, heat treatment of milk before inoculation drives off the oxygen and makes it more suitable for bacterial growth. In

addition to incubation time, incubation temperature and storage time of yogurts appeared to be affected on the cell viability (AkIn et al., 2007).

### **2.15.2 Supplementation of Dairy Products with Polyphenols**

The addition of antioxidants to dairy products can be considered as an emerging trend in the development of functional foods. Among important ingredient groups that can be used for the development of functional dairy products, phytochemicals are preferred as a natural source of antioxidants. The bioavailability of polyphenols in milk is somewhat controversial (Gad & Salam, 2010). Some early studies claimed that maximum antioxidant capacity, and hence better health benefits, could be gained by ingesting milk proteins-polyphenols complex (Hoffman et al., 2001); however, later studies reported reduced bioavailability of polyphenolic compounds after milk ingestion (Serafini et al., 2009).

### **2.15.3 Significance of Polyphenols in Dairy Products**

Milk lipids can undergo oxidative reactions, which may deteriorate their quality. The mechanisms involved include a complex interaction between prooxidants and antioxidants since many antioxidants can be found in milk and several reactions are possible (Lindmark-Mansson & Akesson, 2000; Pandey & Rizvi, 2009). Antioxidants can prevent the formation of free radicals or can scavenge free radicals, hydrogen peroxide or other peroxides. Two antioxidant enzymes, superoxide dismutase and catalase, have been found in milk (Lindmark-Mansson & Akesson, 2000). Non-enzymatic antioxidants can be formed in the animal body or need to be supplied in the feed as essential nutrients (Nimse & Palb, 2015). Another enzyme family with antioxidant functions are the selenium-containing glutathione peroxidases (GSHPx), which catalyse the reduction of different peroxides aided by glutathione or other reducing substrates, including lactoferrin (iron-binding protein), vitamin C (ascorbic acid) and vitamin E (tocopherols and tocotrienols), carotenoids with pro-vitamin A activity, and

flavonoids (Battin & Brumaghim, 2009). Some non-enzymatic antioxidants, including vitamin E and carotenoids, act as free radical scavengers in the lipid phase, whereas vitamin C acts in the aqueous phase. Flavonoids can react in both the lipid and aqueous phases and operate both as radical scavengers and metal ion binders (Lindmark-Mansson & Akesson, 2000).

According to O'Connell and Fox (2001), total polyphenol compounds in milk and other dairy products may be a consequence of several factors including the consumption of particular feed by cattle and the catabolism of proteins by bacteria.

Therefore, this study was conducted to synergistically examine the hypocholesterolemic and antihypertensive effects of developed synbiotic product *in vitro* and *in vivo*. Addition of dietary sources of pomegranate polyphenols due to their potential functional properties has also been considered.

**3. ACID AND BILE TOLERANCE AND  
ANTIHYPERTENSIVE PROPERTIES OF PROBIOTIC  
*LACTOBACILLI* AND *BIFIDOBACTERIA***



### 3.1 INTRODUCTION

Since the early days of probiotics research, *in vitro* screening was a method of choice due to its simplicity and the low cost. Even though some of these tests may seem outdated, they are still in use and can be found in recent reports. The most important advantage of *in vitro* experiments is their ability to screen multiple strains simultaneously (Upadrasta et al., 2011).

Probiotics must be able to reach the GIT alive to exert health benefits and thus, an initial screening of strains based on various stress tolerance situations, especially for non-encapsulated strains directly used in foods, is crucial (Upadrasta et al., 2011). Hence, appropriate *in vitro* tests have been adopted to select strains based on their ability to survive transit throughout the GIT. Survival of potential probiotic bacteria under simulated GIT conditions has been extensively studied over the past decade and strain-specific differences are reported throughout the literature. Following ingestion, probiotics first encounter the harsh conditions of the stomach and they must be able to survive under the extreme acidic conditions and the activity of the digestive enzymes (Hassanzadazar et al., 2012). The acidity of the stomach is known to fluctuate pH from 1-2 to 4-5 after food consumption, but most *in vitro* assays have been developed to select strains that withstand extreme low pH values (Hassanzadazar et al., 2012; Ruiz et al., 2013). Most conventional methodologies include experiments studying the survival of strains in modified growth media, all adjusted to a low pH. However, no similar experiment has been reported for a high value of pH which mimics the slightly alkaline condition of the small intestine, confirming that most probiotic strains are resistant to alkaline conditions (Upadrasta et al., 2011). An acid tolerance test is among the simplest tests that can be performed in order to screen the large number of strains. However, given the unrealistically harsh pH conditions employed during some of these tests may result in the loss of relatively acid-sensitive probiotic candidates (Papadimitriou et al., 2015).

Besides these simplified survival tests, artificial gastric and pancreatic juices have been developed to mimic the *in vivo* conditions (Charteris et al., 1998). These synthetic juices include enzymes pepsin and pancreatin and incubation of probiotic strains in the presence of these juices have been investigated to simulate the time spent by probiotics in the upper and the lower GIT (Lavermicocca et al., 2008). In addition, bile secreted in the small intestine reduces the survival of bacteria by disrupting the cell membrane, inducing protein denaturation, and by damaging DNA (Jarocki et al., 2014). Tolerance to bile salt concentrations of 0.15 to 0.5% has been recommended for probiotics, which is in the range of its physiological concentrations throughout the GIT (Gorbach & Goldin, 1992). Bile tolerance assays are easy to perform, but they may not be reliable selection methods in isolation. In most studies, strains are separately studied for acid or bile tolerance, despite the fact that these two stresses are actually sequential during the transit throughout the GIT, causing cumulative stress damage. The use of non-human bile may also raise questions, as bovine or porcine bile do not have the same impact on microorganisms as human bile (Begley et al., 2005).

Although the use of probiotics has been primarily associated with the improvement of gastrointestinal health, many studies have also illustrated the potential of certain strains of probiotics such as *Lactobacilli* and *Bifidobacteria* in meditating HT via a number of biochemical pathways including RAS involving ACE (Section 2.7.1) (Seppo et al., 2003; Hernández-Ledesma et al., 2004; Geol et al., 2006; Upadrasta & Madempudi, 2016). Inhibition of ACE activity (ACE-I) is thus considered a useful therapeutic approach in HT treatment. The antihypertensive mechanisms of probiotics in the effort to reduce hypertension have been extensively discussed in section 2.7.1.

Despite the strong link between probiotic survival within the host as well as their ACE-I activity and CVD risk in most of the previous studies, probiotic strains have not been extensively studied for their survival ability throughout the GIT and ACE-I activity at the same

time (Papadimitriou et al., 2015). For the development of functional foods, it is necessary to find those probiotic strains that survive stomach acidity and intestinal bile salts. The aim of this study was therefore to evaluate the acid and bile tolerance and antihypertensive properties of 14 probiotic strains and to use the acid and bile tolerant strains with a relatively high ACE-I activity as dietary adjunct in the production of a synbiotic yogurt for future *in vivo* trials.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Source and activation of the cultures

Fourteen frozen probiotic strains from human origin were provided by Dairy Innovation Australia Limited (Werribee, VIC, Australia) (Table 3.1). The stock cultures were stored in 40% glycerol at  $-80^{\circ}\text{C}$ . When required, frozen cultures were thawed at room temperature inside the laminar flow cabinet for 15–20 mins. The thawed cultures were activated by triplicate subculturing in sterile MRS broth (Oxoid, SA, Australia) using 1% inoculums and aerobic incubation at  $37^{\circ}\text{C}$  for 20 h.

**Table 3.1** Probiotic strains used in this study

Genera	Species	Strain	Codes
<i>Lactobacillus</i>	<i>rhamnosus</i>	ASCC 1520	LR 1520
	<i>rhamnosus</i>	ASCC 1521	LR 1521
	<i>rhamnosus</i>	ASCC 2607	LR 2607
	<i>paracasei</i>	ASCC 279	LP 279
	<i>casei</i>	ASCC 270	LC 270
	<i>acidophilus</i>	CSCC 2400	LA 2400
	<i>acidophilus</i>	CSCC 2404	LA 2404
	<i>acidophilus</i>	CSCC 2410	LA 2410
	<i>acidophilus</i>	CSCC 2413	LA 2413
	<i>zeae</i>	ASCC 15820	LZ 15820
	<i>plantarum</i>	ASCC 279	LP 279
<i>Bifidobacterium</i>	<i>longum</i>	CSCC 5022	BL 5022
	<i>longum</i>	CSCC 5089	BL 5089
	<i>bifidum</i>	CSCC 5286	BB 5286

### **3.2.2 Acid tolerance evaluation**

Each of the studied strains was subcultured at least three times prior to experimental use. After final subculture, the incubated cultures were centrifuged at 4,000 g for 20 mins at 4°C. Acid tolerance of the activated cultures was studied by collecting the pellets, dispersing in peptone water and then inoculating (10 % vol/vol) into MRS broth, with pH adjusted to 2.0 with 5 N HCl, and incubated at 37 °C for 2 h to simulate the residence time in the stomach. The bacterial growth was monitored using the plate count method as described by Pereira and Gibson (2002). A 1 mL aliquot was taken every 30 min during incubation for 2 h, and 10-fold serial dilutions were made using peptone water diluent. Samples were plated onto MRS agar and incubated anaerobically at 37°C for 24 h in an anaerobic jar (Becton Dickinson MicrobioLogy Systems®, Sparks, MD., U.S.A.) with an Oxoid CO<sub>2</sub> Gas Generating Kit. Acid tolerance was determined by comparing the final plate count with the initial plate count at time 0. The experiments were triplicated.

### **3.2.3 Bile tolerance**

Two different sources of bile, namely oxgall and taurocholic acid were used to study the bile tolerance of the organisms according to the method described by Gilliland and Walker (1990). Briefly, activated cultures were inoculated (1% vol/vol) into MRS broth at pH 5.0 containing 0.3 % (wt/vol) of oxgall or taurocholic acid and anaerobically incubated at 37°C for 6 h to simulate colonic transit time. The same cultures inoculated in MRS broth without bile salts were used as control. After 0, 3, and 6h of incubation at 37°C, viable counts on MRS agar plates and absorbance of the culture at 620 nm using UV-spectrophotometer (Pharmacia Novaspec II, Cambridge, England) were determined. The experiments were triplicated.

The percentage of growth suppression was calculated and compared to the control (MRS broth without bile salts). In addition, the viability of selected strains in the presence of 0.3 %

(wt/vol) oxgall or taurocholic acid during six hours was measured and compared with the controls. All the experiments were conducted in triplicates.

### **3.2.4 ACE-I Activity**

The ACE-I activity of the selected strains was measured according to Wanasundara et al. (2002) using hippuryl-histidyl-leucine (HHL) as the substrate through high performance liquid chromatography (HPLC). The HPLC standards (Z803898), ACE from rabbit lung ( $\geq 2.0$  units/mg protein; A6778), Hippuryl-histidyl-leucine (HHL) buffer (5 mM Hip-His-Leu in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3; 112003) were purchased from Sigma-Aldrich. The assay was conducted in a Trish buffer (50 mM, pH 8.3) containing 300 mM NaCl and consisted of 50  $\mu$ L of 3.0 mM HHL, 50  $\mu$ L of the ACE solution and 50  $\mu$ L of individual strains grown in MRS. First, the assay was filtered to avoid interference from the bacterial cells present, the serum was transferred into a glass tube and incubated in a water bath for 30 min at 37°C without mixing, then vortexed for 1 min followed by an additional 30 min incubation. Glacial acetic acid (150  $\mu$ L) was added to stop the reaction. The sample thus prepared was kept at -20°C until analysed by HPLC consisting of a Varian 9012 solvent delivery system, a Varian 9100 auto sampler, a reverse-phase column (C18, 250 mm  $\times$  4.6 mm, 5  $\mu$ m; Grace Vydac), a guard column (12 $\mu$ m 10 mm, diameter, Grace Vydac), a Varian 9050 variable wavelength UV-visible absorbance detector set at 228 nm and 730 data modules. Twenty micro-millilitres aliquot of filtered sample was injected into the HPLC column at room temperature (~22°C). The mobile phase used was an isocratic system consisting of 12.5% (vol/vol) acetonitrile (Merck) in MilliQ water, with pH adjusted to 3.0 using glacial acetic acid, at a flow rate of 0.8 mL/min. The control reaction mixture contained 50  $\mu$ L of buffer solution instead of the assay sample and was expected to liberate the maximum amount of Hippuric acid (HA) from the substrate due to uninhibited ACE-I activity.

The amount of HA released from HHL in each assay was determined using the standard curve prepared for HA. The standard solutions of Hippuric acid  $\mu\text{M L}^{-1}$  (Appendix 3.1) was employed to calculate the percentage inhibition of enzyme activity as follow:

$$\% \text{ Inhibition} = \frac{\text{HAc} - \text{HAs}}{\text{HAc}} \times 100$$

Where  $\text{HAc}$  and  $\text{HAs}$  represent the amount of HA in control and in sample respectively.

### 3.2.5 Statistical Analysis

One-way ANOVA was employed to investigate the probiotic strains' acid and bile tolerance, cholesterol removal and antihypertensive abilities ( $p < 0.05$ ). Fisher's test (least significant difference, LSD) was performed to examine the significant difference between means. All statistical analyses were carried out using SAS 9.0 software (SAS Institute Inc, 2008).

## 3.3 RESULTS AND DISCUSSIONS

### 3.3.1 Acid Tolerance

The effect of acid on the viability of selected strains is presented in Table 3.2. All strains showed tolerance towards pH 2.0 for 2h despite variations in the degree of viability. LA 2413, LZ15820, LR 1520, BL 5022 and LA 2404 were the most acid-tolerant strains, with more than  $10^7 \text{ CFU mL}^{-1}$  after incubation for 2h at pH 2.0, while LP276, LA2400 and LPC279 were the most acid-sensitive strains, with only  $10^4$  total  $\text{CFU mL}^{-1}$  after 2h incubation.

The results of this study indicated a greater reduction in total  $\text{CFU mL}^{-1}$  for the strains of *L. acidophilus* and *L. rhamnosus* compared with those of *B. longum* and *B. bifidum* during the first hour of incubation. LA 2404, LR 1520, and LA2413 strains showed the smallest drop in cell population compared with other strains. The population of these strains dropped by 2.9, 3.16 and 3.56  $\text{Log}_{10} \text{ CFU mL}^{-1}$ , respectively, after 120 min incubation at pH 2.0. On the other hand, for the acid-sensitive strains, the viability decreased slowly for the first hour of

incubation followed by a rapid decline at the end of the 2h incubation period. The LP276 strain was found to be the most acid-sensitive strain as its numbers declined 40.63% (6.18 Log<sub>10</sub> CFU mL<sup>-1</sup>) over the 2h incubation period, with most of the decline (4.13 Log<sub>10</sub> CFU mL<sup>-1</sup>) happening in the second hour of incubation.

**Table 3.2:** The viability of the selected *Lactobacillus* and *Bifidobacterium* strains at pH 2.0

Strains <sup>2</sup>	Viable count (Log <sub>10</sub> CFU mL <sup>-1</sup> ) <sup>1</sup>				
	0 min	30 min	60 min	90 min	120 min
<b>Bb52861</b>	10.26±0.152 <sup>a</sup>	9.19±0.08 <sup>a</sup>	8.29±0.03 <sup>a</sup>	6.56±0.02 <sup>b</sup>	4.53±0.03 <sup>bc</sup>
<b>BI5022</b>	10.40±0.04 <sup>a</sup>	9.46±0.15 <sup>a</sup>	8.39±0.22 <sup>a</sup>	6.53±0.04 <sup>b</sup>	4.29±0.19 <sup>bc</sup>
<b>BI5089</b>	10.30±0.18 <sup>a</sup>	8.26±0.06 <sup>a</sup>	7.55±0.14 <sup>a</sup>	6.11±0.52 <sup>b</sup>	5.24±0.17 <sup>bc</sup>
<b>LA2400</b>	10.23±0.16 <sup>a</sup>	8.71±0.11 <sup>a</sup>	7.14±0.59 <sup>a</sup>	6.18±0.04 <sup>b</sup>	5.59±0.03 <sup>bc</sup>
<b>LA2404</b>	10.46±0.28 <sup>a</sup>	9.70±0.05 <sup>a</sup>	8.55±0.57 <sup>a</sup>	7.99±0.18 <sup>a</sup>	7.55±0.16 <sup>a</sup>
<b>LA2410</b>	10.09±0.06 <sup>a</sup>	9.07±0.33 <sup>a</sup>	8.40±0.10 <sup>a</sup>	7.30±0.18 <sup>a</sup>	5.21±0.03 <sup>bc</sup>
<b>LA2413</b>	10.65±0.03 <sup>a</sup>	10.20±0.02 <sup>a</sup>	9.07±0.12 <sup>a</sup>	8.68±0.19 <sup>a</sup>	7.00±0.08 <sup>a</sup>
<b>LC290</b>	10.35±0.19 <sup>a</sup>	8.35±0.19 <sup>a</sup>	6.47±0.09 <sup>b</sup>	5.93±0.14 <sup>b</sup>	5.18±0.07 <sup>b</sup>
<b>LP276</b>	10.41±0.28 <sup>a</sup>	9.22±0.60 <sup>a</sup>	8.36±0.09 <sup>a</sup>	6.37±0.08 <sup>b</sup>	4.23±0.03 <sup>bc</sup>
<b>LPC279</b>	10.13±0.55 <sup>a</sup>	8.28±0.07 <sup>a</sup>	7.89±0.63 <sup>a</sup>	6.94±0.13 <sup>b</sup>	5.93±0.15 <sup>b</sup>
<b>LR1520</b>	10.62±0.12 <sup>a</sup>	9.91±0.12 <sup>a</sup>	8.70±0.03 <sup>a</sup>	8.33±0.02 <sup>a</sup>	7.46±0.10 <sup>a</sup>
<b>LR1521</b>	10.05 ± 0.64 <sup>a</sup>	9.41 ± 0.27 <sup>a</sup>	7.64 ± 0.59 <sup>a</sup>	6.55 ± 0.18 <sup>bc</sup>	5.67 ± 0.24 <sup>bc</sup>
<b>LR2607</b>	10.13 ± 0.58 <sup>a</sup>	8.27 ± 0.75 <sup>a</sup>	7.60 ± 0.03 <sup>a</sup>	6.73 ± 0.32 <sup>b</sup>	5.77 ± 0.17 <sup>bc</sup>
<b>LZ15820</b>	10.18 ± 0.64 <sup>a</sup>	8.59 ± 0.62 <sup>a</sup>	7.67 ± 0.03 <sup>a</sup>	6.26 ± 0.04 <sup>bc</sup>	6.15 ± 0.71 <sup>b</sup>

<sup>1</sup>Bacterial codes are presented in Table 3.1.

<sup>abc</sup> Means in the same column followed by different superscript letters are significantly different ( $P < 0.05$ ).

<sup>2</sup> Results are expressed as mean ± SEM; each data point is the average of 3 replicate measurements (n=3).

The time from food entering the stomach to the time that chyme is released from the stomach has been estimated to be approximately 90 min (Berada et al., 1991). Survival at pH between 2.0 and 3.0 for 2h and in bile concentration of 1000 mg L<sup>-1</sup> is considered optimal for acid and

bile tolerance of probiotic strains (Usman & Hosono, 1999). In this study, all organisms tolerated 0.30% oxgall, which was three times higher than the suggested level for probiotic organisms (Usman & Hosono, 1999).

Some strains used in this study include LA 2413, LZ15820, LR 1520, BL 5022 and LA 2404 showed excellent survival at pH 2.0 after 2h incubation, whereas, Manini et al. (2016) who studied the acid tolerance of 16 LAB at pH 2.0 after 2h reported zero survival for 7 out of 16 strains at the end of a 2h incubation period. Liong and Shah (2005) studied the acid tolerance of eleven probiotics (four strains of *L. acidophilus* and seven strains of *L. casei*) at pH 2.0 during 120 min and reported varying levels of viability at pH 2.0 after 2 h incubation. *Lactobacillus acidophilus* ATCC 4357, *L. acidophilus* ATCC 4962, *L. casei* ASCC 290 and *L. casei* ASCC 292 survived best under the acidic conditions, while viability of strains ASCC 1520, ASCC 1521, ASCC 279, ATCC 15820 and CACC 2607 was greatly reduced. *Lactobacillus acidophilus* strains survived better under acidic conditions than *L. casei*.

Acid tolerance of bacteria is important not only for withstanding gastric stresses, but also a prerequisite for their use as dietary adjuncts. Furthermore, acid tolerance enables strains to survive for longer periods of time in high acid carrier-food without a larger reduction of bacteria in human's gut (Shehata et al., 2016).

The mechanisms of acid tolerance of these strains have been reported in several studies. Waddington et al. (2010) demonstrated that the response of *B. longum* to acid stress involved the maintenance of pH homeostasis by activation of the plasma H<sup>+</sup>-ATPase and proton pump and production of NH<sub>3</sub>.

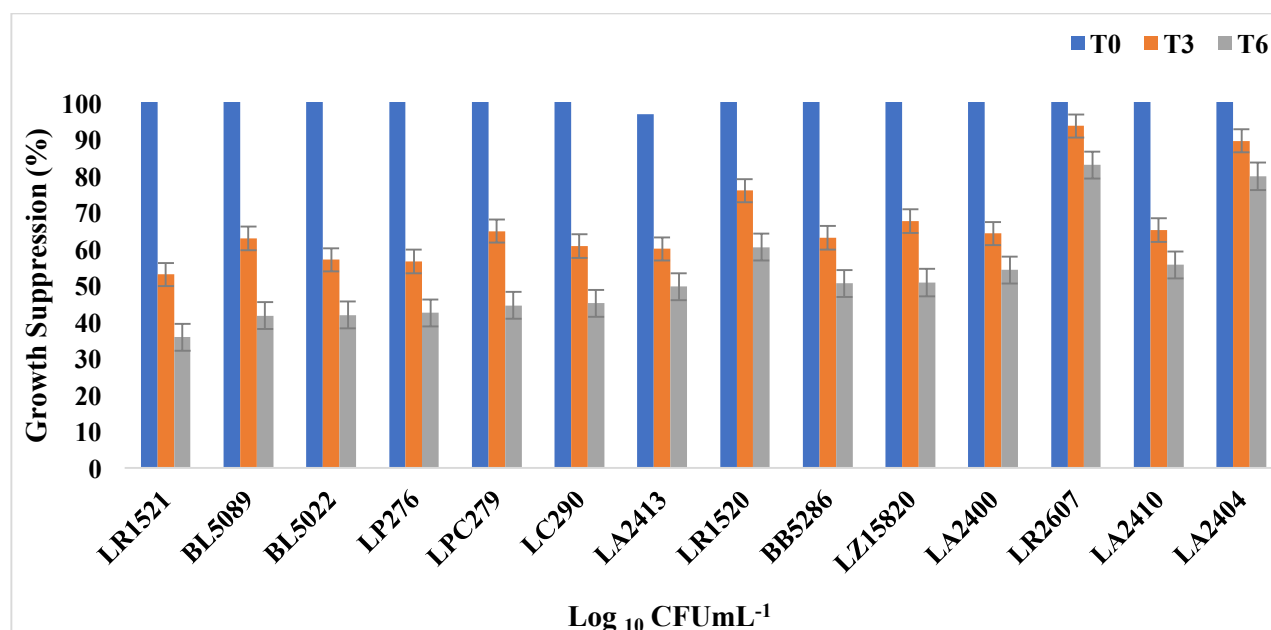
Mechanisms underlying acid tolerance used by Gram-positive bacteria are complex. Several mechanisms have been postulated for acid tolerance by LAB, including the maintenance of pH homeostasis and intracellular pH regulation (Matsui & Cvitkovitch, 2010), protecting DNA and protein (Cappa et al., 2005), neutralizing the excessive H<sup>+</sup> (Tonon & Lonvaud-Funel,



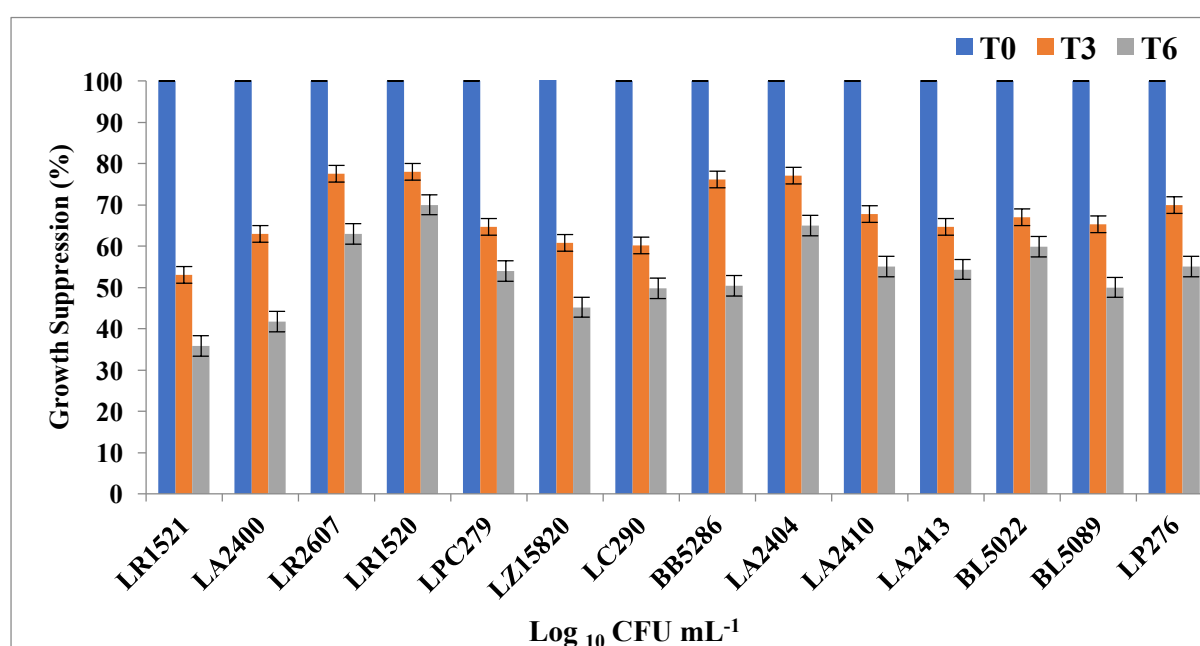
2000), as well as signal communication (Matsui & Cvitkovitch, 2010). Thus, identification of all underlying mechanisms to improve the acid resistance of LAB in industrial applications is necessary.

### **3.3.2 Bile Tolerance**

The viability of selected LAB grown in MRS supplemented with 0.3% of taurocholic acid and oxgall during six hours of incubation at 37°C are measured and compared with the control (Figures 3.3 and 3.4, respectively). Although, most strains showed a significant decline in numbers ( $P < 0.05$ ) after the first 3h of incubation, LR2607, LA2404 and LR1520 strains did not reveal any significant growth suppression during the same period (Figure 3.1 and 3.2). The growth suppression after the first 3h of incubation for all strains ranged from 1.0% to 47%, whereas after 6h the suppression became more pronounced (30% to 64%). In the presence of taurocholic acid, bacterial strains LR2607, LA 2404, and LR 1520 showed the lowest percentage of growth suppression, while LR1521, BL5089, BL5022 had the highest percentage of growth suppression. Figure 3.2 shows that bacterial strains had the similar trend during 6 h of incubation at 37°C in MRS supplemented with oxgall. LR1520, LA2404, and LR2607 were the most tolerant towards oxgall, while LR1521, LA 2400, LZ15820 were the least tolerant strains (Figure 3.2). A low growth suppression indicates the ability of the strains to survive the *ca.* 6 h transit time through the GIT (Lye et al., 2010).



**Figure 3.1** The viability of the selected probiotics in MRS supplemented with taurocholic acid (0.30% w/v), incubated for 6 h at 37°C. Results are expressed as mean  $\pm$  SEM; each data point is the average of 3 replicate measurements (n=3).



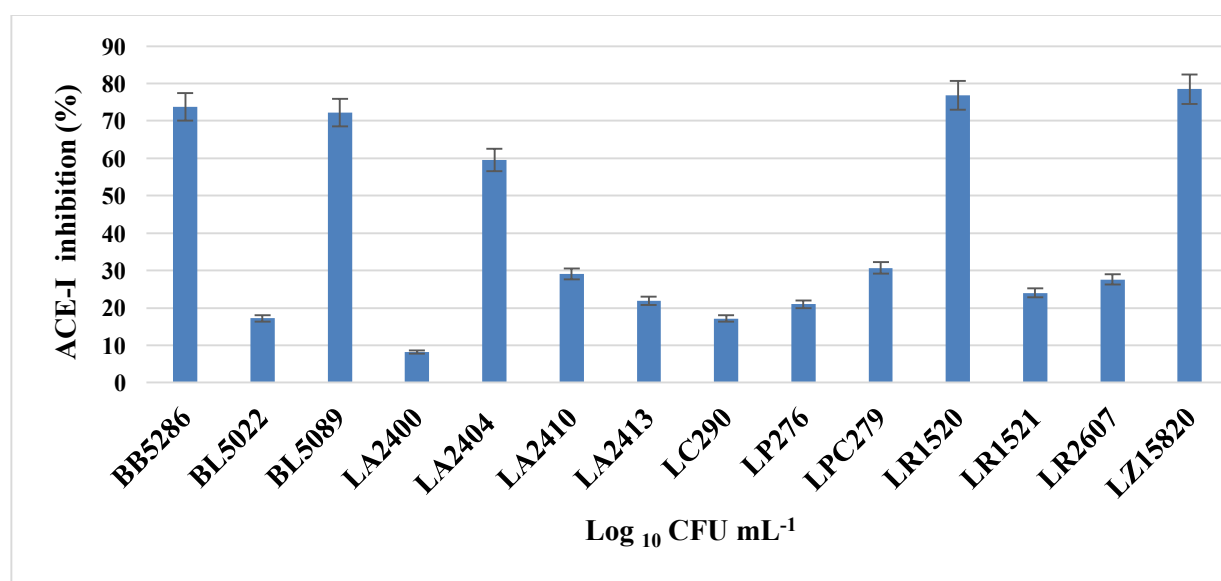
**Figure 3.2** The viability of the selected probiotics in MRS supplemented with oxgall (0.30% w/v), incubated for 6 h at 37°C. Results are expressed as mean  $\pm$  SEM; each data point is the average of 3 replicate measurements (n=3).

Liong and Shah (2005) examined the bile tolerance of eleven probiotic strains (four strains of *L. acidophilus* and seven *L. casei*) and reported that all selected strains could tolerate the presence of bile, with greater tolerance towards oxgall compared to taurocholic acid. Different growth rates observed in the presence of selected bile sources suggested that taurocholic acid provided more growth suppression towards LAB strains than oxgall and glycocholate (Liong & Shah, 2005). Devi et al. (2015) tested the resistance to acid (pH 2 and 3) and bile salts (0.1, 0.3 and 0.5% w/v of oxgall) of seventy-one LABs isolated from infant faecal material and dairy samples during 48 h of incubation. Thirty-eight strains among the seventy-one tested, were reported to have high survival rates of 81-93% after 24 h and 57-86% after 48 h of incubation. Tolerance to bile salts is a prerequisite for colonisation and metabolic activity of bacteria in the small intestine of the host (Shehata et al., 2016). This will help LAB to reach the small intestine and colon and contribute to balancing the intestinal microflora. All the tested strains exhibited bile tolerance with varying degrees. Among the tested LAB isolated in the present study, LA2404, LR1520 and LR2607 demonstrated the highest bile salt tolerance.

### **3.3.3 Inhibition of ACE-I activity**

The impact of individual strains on inhibition of ACE-I activity in MRS was measured and compared with the cell-free control MRS. The ACE-I activity inhibition effect ranged from a maximum of 78.5% to a minimum of 8.2% for all tested strains (Figure.3.5). Analysis of variance showed that ACE-I activity was significantly different among the strains ( $P < 0.05$ ) thus it was strain-dependent. Strains LZ15820, LR1520, BB5286 and BL5089 provided the highest ACE-I activity among the selected strains (78.5, 76.9, 73.8 and 72.2%, respectively), followed by LA 2404 which showed a relatively reasonable antihypertensive property of 60% (Figure. 3.5). These results may be attributed to the strong proteolytic enzyme system of these

strains that enable them to produce more metabolites that act as inhibitors to the angiotensin-converting enzyme.



**Figure 3.3** Inhibition of ACE-I activity (%) by selected LAB. Results are expressed as mean  $\pm$  SEM; each data point is the average of 3 replicate measurements (n=3).

Similarly, Yu et al. (2009) investigated the ACE-I activity of 6 *Lactobacillus* sp. isolated from Kimchi using spontaneously hypertensive rats. Most tested strains showed the capacity for ACE-I inhibition, however, it was significantly ( $P < 0.05$ ) different among the strains. The strongest ACE-I activity was shown by *L. plantarum* LG 7, 8 and 42. The authors showed that after four weeks, the SBP in *L. L. plantarum* LG 42 group ( $10^9$  Log<sub>10</sub> CFU mL<sup>-1</sup>) was approximately 27% lower than that of the control group.

Given that a high level of BP has been associated with the risk of CVD, those strains with the strongest ACE-I activity have attracted much attention in the formulation of functional foods (Roberfroid, 2000; Zubillaga et al., 2001). The initial objective of this study was to identify the strains of probiotics, from the fourteen selected strains, with maximum acid and bile tolerance and antihypertensive properties through *in vitro* assays. Based on the reported results, two strains were identified as the most suitable, *L. acidophilus* ASCC 2404 and *L.*

*rhamnosus* ASCC 1520 for application in the formulation of a synbiotic yogurt for use in subsequent human trials to further document probiotic health benefits. The findings of a human study is reported in chapter 6.

### **3.4 CONCLUSION**

All strains of *Lactobacilli* and *Bifidobacteria* studied survived the acidic conditions and bile concentrations and showed hypotensive properties to a variable extent. However, the maximum acid and bile tolerant and ACE-I activity were observed in *L. acidophilus* CSCC 2404 and *L. rhamnosus* ASCC 1520 strains and therefore have been selected for use in the development of a synbiotic yogurt used in our human study.

#### **4. CHOLESTEROL REDUCTION MECHANISMS AND FATTY ACID COMPOSITION OF CELLULAR MEMBRANES OF PROBIOTIC *LACTOBACILLI* AND *BIFIDOBACTERIA* <sup>1</sup>**

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<sup>1</sup> This section has been published in Journal of Functional Foods: Miremadi, F., Ayyash, M., Sherkat, F., Stojanovska, L. 2014. “Cholesterol reduction mechanisms and fatty acid composition of cellular membranes of probiotic Lactobacilli and Bifidobacteria”. *Journal of Functional Foods* 9, 295-305.

## 4.1 INTRODUCTION

Epidemiological studies have shown that a higher than normal serum cholesterol level is strongly associated with the development of cardiovascular disease (CVD), which is one of the leading causes of death and disability in developed countries (Manson et al., 1992; Kunnen & Van Eck, 2012). The World Health Organisation (WHO) has predicted that CVD will remain the major cause of death by 2030, and will affect almost 23 million people (Anon., 2009). In clinical trials, it has been reported that for each 1 mmol of higher than normal cholesterol level ( $> 5.2 \text{ mmol L}^{-1}$ ), the risk of coronary heart disease increases by 35 %, whilst a small reduction in serum cholesterol of 1 % reduces the risk by 2 - 3 % (Manson et al., 1992). Moreover, oxidation of cholesterol leads to the formation of arterial plaques and, hence, is a leading cause of cardiovascular disease (Shahidi, 2007). In the human body there are two main sources of cholesterol; (i) internal biosynthesis by the liver, and, (ii) from the diet, mainly from foods containing animal fats (Lecerf & De Lorgeril, 2011). Although drugs such as statins effectively decrease cholesterol, side effects are commonly reported, leading to patients seeking alternative options such as, exercise, weight loss, supplements and diet (Bliznakov, 2002). Further, the use of conventional cholesterol-lowering drugs adds additional high costs to the individual as most often, anti-cholesterol drugs are taken for life.

The bioactive components of functional foods and natural health products are responsible for their efficacy in health promotion and disease prevention therefore, functional foods have been recommended as a favourable dietary approach to reduce total cholesterol, especially for those patients with borderline blood cholesterol levels who do not require cholesterol-lowering medications (Shahidi, 2004; Chen et al., 2011; Wang et al., 2012). Moreover, functional foods are available at affordable prices with a lower possibility of side effects (Monteagudo-Mera et al., 2012; Vidhyasagar & Jeevaratnam, 2013). In addition, reports suggest that functional foods containing probiotics reduce the risk of coronary heart disease

and therefore, the characterisation of the active ingredients and the type and number of the probiotics is important (Chen et al., 2011; Shahidi, 2012).

Probiotics have been defined as ‘live microorganisms’ which, when taken in adequate amounts in the diet, deliver health benefits to the host (Vidhyasagar & Jeevaratnam, 2013). However, the selection of potential probiotic strains which are effective in the gastrointestinal tract is a significant challenge (Gibson et al., 2000). Strain selection has generally been based on *in vitro* tolerance to physiologically relevant gastrointestinal stressors such as low pH and varying types and concentrations of bile salts, which may decrease their viability (Gibson et al., 2000; dos Santos Leandro et al., 2013). In addition, the ability of probiotic strains to hydrolyse bile salts has often been included among the criteria for probiotic strain selection. Mann and Spoerry (1974) were the first to claim that the consumption of milk fermented with *Lactobacillus acidophilus* reduced serum cholesterol in hypercholesterolaemic African subjects. Since then, the hypocholesterolaemic effect of fermented dairy products has been investigated in feeding studies in humans (Park et al., 2006) or animal models (Danielson et al., 1989; Liong & Shah, 2006). These studies suggest that some strains of *Lactobacilli* could lower total cholesterol and low-density lipoprotein (LDL-C), resulting in a beneficial effect on serum cholesterol levels.

Although the exact mechanisms of serum cholesterol reduction by probiotic bacteria are unclear, several potential cholesterol removal mechanisms have been proposed (Lye et al., 2010). One such mechanism is deconjugation of bile salts by probiotic bacteria producing bile salt hydrolase (BSH). Probiotics are able to hydrolyse glycin- or taurin- conjugated bile salts into amino acid residues and free bile salts (Reynier et al., 1981; Corzo & Gilliland, 1999). Conjugated bile salts are usually re-circulated through enterohepatic circulation, while deconjugated bile salts are less soluble and are excreted in the faeces (Liong & Shah, 2005). The excreted bile salts are replaced by new bile salts formed from cholesterol in the blood



stream. Thus, the more bile salt excreted, the more cholesterol is removed from the blood stream (Walker & Gilliland, 1993). Some studies have also reported that low pH would favour the secretion of BSH (Corzo & Gilliland, 1999). Milk lactose is a potent energy source for *Lactobacilli* that produces lactic acid and short chain fatty acids as the main metabolites, leading to a reduced pH. Therefore, fermented dairy products such as yogurt could enhance BSH production.

A number of *in vitro* studies have shown that some strains of *Lactobacillus acidophilus* secrete BSH and deconjugate bile salts (Walker & Gilliland, 1993); however, others have found no relationship between the amount of *in vitro* cholesterol removal and the degree of bile salt deconjugation (Hill & Drasar, 1968; Dambekodi & Gilliland, 1998). These conflicting results lead to the proposal of another cholesterol removal mechanism, which may be associated with cholesterol assimilation from the media into the cellular membranes of probiotic bacteria during growth (Lye et al., 2010). Indeed, it has been noted that cholesterol increases the tensile strength of mycoplasma cellular membrane and permits their survival and growth without the protection of the cell walls (Razin, 1967). Likewise, cholesterol removal from media by *Lactobacillus Lactis* subsp. *Lactis* biovar *diacetyllactis* N7 strain is incorporated into the cellular membrane resulting in altered fatty acid composition of the bacterial cell (Kimoto et al., 2002). The presence of mono-unsaturated fatty acids increases cholesterol uptake by epithelial cells in the small intestine of rats, and the uptake is inhibited in the presence of saturated fatty acids (Safonova et al., 1994).

It has been reported that even heat-killed cells of some probiotic strains can remove cholesterol from the media due to cholesterol attachment to the cellular membrane (Kimoto et al., 2002; Tok & Aslim, 2010), even though the degree of removal is significantly less as compared to growing probiotics. Thus, it appears that some probiotic strains can remove

cholesterol from media both by binding of cholesterol to dead cells and by the uptake of cholesterol into living cells during growth. The mechanism by which cholesterol is removed has been studied; however, only one bacterium has been evaluated at a time. In addition, some of the previous studies showed that cholesterol removal is highly dependent on strain growth conditions (Pereira & Gibson, 2002). Hence, this study evaluated the cholesterol removal ability of fourteen strains of *Lactobacilli* and *Bifidobacteria* via several cholesterol removal mechanisms including, cholesterol assimilation, incorporation of cholesterol into cellular membranes, binding of cholesterol to cells and bile salt deconjugation. As a result, the most beneficial strains will be used as adjunct cultures in the development of synbiotic foods for *in vivo* cholesterol reduction studies.

## **4.2 MATERIAL AND METHODS**

### **4.2.1 Source and maintenance of culture**

Fourteen frozen probiotic strains from human origin provided by Dairy Innovation Australia Limited (Werribee, VIC, Australia) was used in this study. These included three *Lactobacillus rhamnosus* strains ASCC 1520 (LR 1520), ASCC 1521 (LR 1521), and ASCC 2607 (LR 2607); *Lactobacillus paracasei* ASCC 279 (LP 279), *Lactobacillus casei* ASCC 270 (LP 270), four *Lactobacillus acidophilus* strains: CSCC 2400 (LA 2400), CSCC 2404 (LA 2404), and CSCC 2410 (LA 2410), and CSCC 2413 (LA 2413), *Lactobacillus zeae* ASCC 15820 (LZ 15820), *Lactobacillus plantarum* ASCC 279 (LP 279), two *Bifidobacterium longum* strains: CSCC 5022 (BL 5022) and CSCC 5089 (BL 5089), and *Bifidobacterium bifidum* CSCC 5286 (BB 5286). The stock cultures were stored in 40 % glycerol at -80 °C. When required, the frozen cultures were thawed at room temperature inside the laminar flow cabinet for 15-20 min. The cultures were activated by triplicate subculturing in sterile de Mann,

Rogosa, Sharpe (MRS) broth (Oxoid, SA, Australia) using 1% inoculum and incubation at 37 °C for 20 h and stored at 4 °C between transfers.

#### **4.2.2 Measurement of cholesterol assimilation**

Thirty milligrams of water-soluble cholesterol (polyoxyethanyl-cholesterol sebacate) (Sigma, St. Louis, MO, USA) were dissolved in 10 mL Milli-Q water and filter-sterilized using 0.45 µm filter (Millipore, Corp., Bedford, MA, USA) to obtain a stock solution of cholesterol. The sterilised MRS broth (Oxoid) containing 0.30 % bile salt oxgall (Sigma) and 100 µL/mL cholesterol stock solution was inoculated with 1 % activated probiotic cultures and incubated at 37 °C for 24 h. The inoculated MRS was sampled at 6, 12, and 24 h intervals during incubation time followed by centrifugation at  $4000 \times g$  for 20min at 4 °C. The cholesterol content of the supernatant was determined using a modified colorimetric method as previously described by Rudel and Morris (1973) with minor modifications as detailed in Liong and Shah (2005). Briefly, 1 mL of the supernatant was mixed with 1 mL KOH (33 %, w/v) and 2 mL 96 % ethanol. The mixture was vortexed for 1 min followed by incubation at 37 °C for 15 min, and cooled to room temperature. Upon cooling, 2 mL of Milli-Q water and 3 mL of hexane were added to the mixture followed by vortexing for 1 min. The mixture was then allowed to settle until the separation of the two layers. The upper hexane layer was collected and evaporated under nitrogen gas. Two millilitres of o-phthalaldehyde reagent (50 mg OPA dissolved in 100 mL glacial acetic acid; Sigma) were added and vortexed for 1 min to dissolve the residues. To this, 0.5 mL of sulphuric acid (98 %; Sigma) was added and vortexed for 1 min followed by resting for 10 min at room temperature before measuring the absorbance at 550 nm using UV-spectrophotometer (Pharmacia Novaspec II, Cambridge, England). The cholesterol concentration was read off a standard curve prepared using the cholesterol stock solution. All experiments were conducted in triplicate and assayed twice ( $n = 6$ ). The ability of

probiotics to assimilate cholesterol was expressed as the percentage of cholesterol removed at each incubation interval as follow:

$$\% \text{ of cholesterol removed} = \frac{100 - \text{residual cholesterol at each incubation interval}}{100} \times 100$$

#### **4.2.3 Bacterial growth**

Growth of all probiotic strains in the presence or absence of cholesterol was assessed during incubation time at 37 °C for 24 h. Sterilized MRS broth supplemented with 0.30 % oxgall and 100 µg/mL cholesterol was inoculated with 1 % activated strain and sampled at 6, 12, and 24 h. Bacterial growth was monitored indirectly via measuring cholesterol assimilation using UV-spectrophotometer (Pharmacia Novaspec II, Cambridge, England) at 620 nm.

#### **4.2.4 Cholesterol removal by non-growing (resting) and dead cells**

To measure the ability of non-growing cells to remove cholesterol, freshly prepared MRS broth containing 0.3 % oxgall was inoculated with each probiotic strain and incubated for 24 h at 37 °C. Cells were harvested by centrifuging at 4000 x g (Microspin 24, Sorvall Instruments, Melbourne, Australia) at 4 °C for 20 min and washed twice with sterile Milli-Q water. To prepare heat-killed cells, cell pellets were suspended in 10 mL of Milli-Q water and autoclaved at 121 °C for 15 min. Heat-killed cells were further suspended in MRS broth supplemented with 0.3 % (w/v) oxgall acid and 100 µL/mL of water-soluble cholesterol (polyoxyethanyl-cholesterol sebacate) (Sigma, St. Louis, MO, USA) and incubated for 24 h at 37 °C. To prepare non-growing (resting) cells, cell pellets were suspended in 10 mL sterile phosphate buffer (0.05 M, pH 6.8) containing 0.3 % (w/v) oxgall and 100 µL/mL of water-soluble cholesterol and incubated for 24 h at 37°C. Following incubation, the mixtures were centrifuged (4000 x g at 4 °C for 20 min) and cholesterol concentrations in the supernatants

were measured using the spectrophotometric method described above. All experiments were conducted in triplicate and assayed twice (n = 6).

#### **4.2.5 Deconjugation of bile salts**

The amount of bile salts (sodium glycocholate, sodium taurocholate, and a mixture of sodium glycocholate and sodium taurocholate) deconjugated by *Lactobacilli* and *Bifidobacteria* was determined using BSH activity and liberated free cholic acid as parameters.

##### *4.2.5.1 Free cholic acid*

The concentration of cholic acid was determined using UV-spectrophotometer as previously described (Liong & Shah, 2005). Ten millilitres aliquots of MRS broth were supplemented with 6 mM sodium glycocholate, 6 mM sodium taurocholate, and a mixture of 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate. The mixtures were then individually inoculated with 1 % activated probiotic followed by incubation for 24 h at 37 °C. Individual bile salts were added at 6 mM each, as this dose closely mimics that prevailing in the human small intestine (Brashears et al., 1998). In addition, bile mixtures were added at 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate, as this resembles the molar ratio of the two salts in human bile (Pilch et al., 2012). Following incubation, media were adjusted to pH 7.0 with 1 M NaOH followed by centrifugation at 4000 × g at 4 °C for 20 min. The supernatant was collected and adjusted to pH 1.0 with 5M HCl. One millilitre from the adjusted supernatant was mixed with 2 mL ethyl acetate (Merck), vortexed for 1 min and allowed to settle for layer separation. The ethyl acetate layer was transferred into glass tubes and evaporated under nitrogen at 60 °C. One millilitre of 0.01 M NaOH was added to dissolve the residue followed by 1 mL 1 % (v/v) furfuraldehyde (Sigma) and 1 mL 8 M sulphuric acid. The mixture was further vortexed and heated at 65 °C for 10 min. Glacial acetic acid was added upon cooling and the concentration of cholic acid was measured using UV-spectrophotometer

(Pharmacia Novaspec II, Cambridge, England) at 660 nm. The amount of cholic acid released was determined using external cholic acid standard curve (Sigma). All experimental wells were in triplicate and repeated at least twice ( $n = 6$ ).

#### 4.2.5.2 *Bile salt hydrolysis*

The BSH activity was measured according to Tanaka et al. (2000). Briefly, activated probiotics were individually cultured in MRS broth and incubated for 24 h at 37 °C. Cell pellets were harvested by centrifugation at  $4000 \times g$  for 20 min followed by two washes in a phosphate buffer (0.1 M, pH 7.0), and resuspended in the phosphate buffer to obtain a cell suspension with an optical density of 1.0 at 600 nm. An aliquot of 300  $\mu$ L Cellytic<sup>TM</sup> B 10  $\times$  concentrate (Sigma) was added to the cell suspension for 3 min in order to rupture the cell walls, followed by centrifugation at  $4000 \times g$  at 4 °C for 10 min. According to Sigma instructions, Cellytic<sup>TM</sup> B 10  $\times$  concentrate has no adverse effect on the bacterial enzymes after rupturing or on the subsequent assays (Tanaka et al., 2000). An aliquot of 0.1 mL of the supernatant was mixed with 1.8 mL of 0.1M sodium phosphate buffer (pH 6.0) and 0.1 mL of conjugated bile salts (6 mM sodium glycocholate, 6 mM sodium taurocholate, a mixture of 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate). The mixture was incubated for 30 min at 37 °C, followed by the addition of 0.5 mL trichloroacetic acid (15 % (w/v); Sigma) to stop the enzymatic reaction. The mixture was then centrifuged at  $4000 \times g$  at 4 °C for 5 min. A 0.2 mL aliquot of the supernatant was mixed with 1 mL Milli-Q water and 1 mL ninhydrin reagent prepared as follows: 1 g ninhydrin (Sigma) in 100 mL Milli-Q water (1% w/v). A 0.5 mL aliquot of prepared 1 % ninhydrin reagent was mixed with 0.2 mL of 0.5 M citrate buffer (pH 5.5) and 1.2 mL of 30 % (v/v) glycerol. The mixture was vortexed for 1 min and heated for 14 min at 100 °C and subsequently cooled to 25 °C. The absorbance at 570 nm was read using UV spectrophotometer (Pharmacia Novaspec II, Cambridge, England). The concentration of

the amino acids released was determined by external standard curve of glycine (Sigma). One unit of BSH activity (U/mL) was defined as the amount of enzyme that liberated 1 mmol of amino acid from the substrate per min under the assay conditions. Protein concentrations were measured according to Bradford (1976) using Bradford reagent (Sigma). Specific activity of the organism was expressed as units (U) of BSH activity per mg of protein. All experimental wells were triplicate and assayed twice ( $n = 6$ ).

#### *4.2.5.3 Fatty acids profile of cellular membrane*

Fatty acid composition of the cellular membrane may impact cholesterol uptake by epithelial cells. Hence, the fatty acid composition of cellular membranes were analysed as previously described (Murga et al. (2000) and (Liong and Shah (2005)). Freshly prepared MRS broth containing 0.3 % oxgall and 100  $\mu$ L water-soluble cholesterol was inoculated with each probiotic (1 %) and incubated for 24 h at 37 °C. Cells were harvested after the incubation period by centrifuging at  $4000 \times g$  (Microspin 24, Sorvall Instruments, Melbourne, Australia) for 20 min at 4 °C. The cell pellets were mixed with 1 mL methanol/chloroform (2:1, v/v), followed by vortexing for 2 min, standing for 24h at 4 °C and centrifuged at  $10,000 \times g$  for 10 min at 4 °C. The supernatant was then collected and the cell pellet was washed twice with 1 mL of methanol/chloroform/Milli-Q water (2:1:0.8 v/v/v). The supernatant was pooled followed by vortexing for 1 min. To this, 1 mL each of Milli-Q water and chloroform were added, followed by vortexing for 2 min. The mixture was allowed to separate and the bottom chloroform layer was collected and evaporated. The lipid residue was dissolved in 0.5 mL of hexane and converted into fatty acid methyl esters with the addition of 100  $\mu$ L of methanoic KOH (2 M). Hydrochloric acid (2 M) was added until methyl orange indicator changed to pink and the mixture was allowed to settle. The organic layer (10  $\mu$ L) was collected and injected into a gas chromatography (Varian Star 3400 CX, Walnut Creek, CA, USA) system that was equipped

with a flame ionization detector. A stainless-steel column (30 m × 0.25 mm) packed with 70 % cyanopropyl polysilphenylene siloxane (SGE Analytical Science Pty Ltd, Ringwood, VIC, Australia) was used. The oven temperature was gradually increased from 100 to 225 °C at a rate of 5 °C/min. The temperatures of the injector and detector were 260 and 280 °C, respectively. The carrier gas was helium at a flow rate of 3 mL/min. The split ratio of 1:100 and the injection volume of 1 µL were applied. The concentration of fatty acid methyl esters in the samples was determined using standard fatty acids purchased from (Sigma). All experiments were conducted in triplicates and assayed in duplicates.

#### **4.2.6 Statistical analysis**

One-way ANOVA was employed to investigate the effect of probiotic strains on cholesterol assimilation, bile salt deconjugation, fatty acid composition and BSH activities ( $p < 0.05$ ). Fisher's test (least significant difference, LSD) was performed to examine the significant difference between means. Two-way ANOVA was carried out to examine the effect of probiotic strains × incubation time interaction on variables. Pearson's correlation was applied to investigate the relationship between the probiotic growth and cholesterol assimilation. All statistical analysis were carried out using SAS 9.0 software (SAS Institute Inc, 2008).

### **4.3 RESULTS AND DISCUSSION**

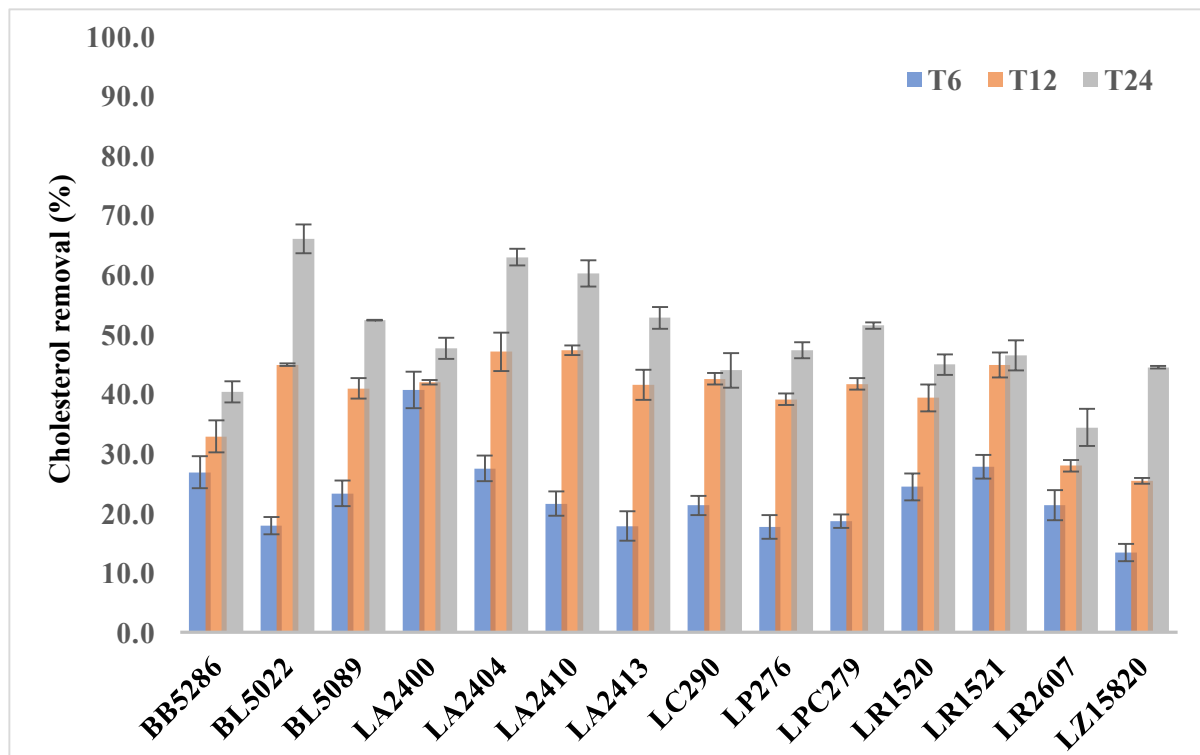
#### **4.3.1 Probiotics remove cholesterol *in vitro***

The removal of cholesterol by fourteen probiotic bacterial strains grown in MRS supplemented with 0.3 % oxgall is shown in Figure 4.1. All probiotics were able to assimilate cholesterol at varying degrees. Oxgall (dehydrated fresh bile) closely represents bile of the human gut and a 10 % solution is equivalent to fresh bile (Kimoto et al., 2002). Analysis of variance showed that the percentage of cholesterol assimilation varied significantly ( $p < 0.05$ )



amongst different probiotics at the same incubation times (6, 12, and 24 h). The cholesterol assimilation ranged from 13 - 40 %, 25 - 47 % and 34 - 65 % at 6 h, 12 h, and 24 h of incubation, respectively (Figure 4.1). In particular, probiotic strains BL5022, LA2404, LA2410 and BB5286 showed significantly ( $p < 0.05$ ) higher cholesterol removal ability (65, 62, 60 and 52 %, respectively) at 24 h of incubation as compared to the other strains. These results are in accordance with other studies, which have reported cholesterol assimilation properties for *Lactobacillus* and *Bifidobacteria* species (Pereira & Gibson, 2002; Wang et al., 2012). For all probiotics, the amount of cholesterol removal increased significantly ( $p < 0.05$ ) as the incubation time increased. A significant negative correlation exists between the residual cholesterol in the MRS broth and the growth of BL5022, LA2404, LA2410 and BB5286 strains with respective correlation coefficients ( $r$ ) of -0.808, -0.760, -0.776 and -0.811 (Figure 4.2).

Cholesterol assimilation patterns indicate that cholesterol removal is growth-associated. Most strains exhibited enhanced growth in the presence of cholesterol, indicating that cholesterol may influence their growth. Cholesterol assimilation in the presence of oxgall showed a good relationship ( $r^2 = 0.760 - 0.808$ ) with bile tolerance of the strain studied (Figure 4.1). Strains with the greatest tolerance towards sodium glycocholate showed higher overall cholesterol assimilation in the presence of oxgall, whereas those with higher inhibition towards taurocholic acid produced lower cholesterol assimilation (Figure 4.2). The lower cholesterol removal ability observed in LR2607 and BB5286 strains (Figure 4.1) may be due to differences in their cell wall composition that makes them unable to bind cholesterol, and also may result from their lower tolerance towards sodium glycocholate and oxgall (Kimoto et al., 2002).



**Figure 4.1:** Cholesterol removal (%) of the 14 probiotic bacteria incubated in MRS supplemented with 100 µg/mL water-soluble cholesterol and 0.3 % oxgall for 24 h at 37 °C.

#### 4.3.2 Cholesterol removal by growing, resting and dead probiotics

The ability of fourteen probiotic strains, either growing or non-growing (resting or dead) to remove cholesterol was assessed. The removal of cholesterol varied significantly ( $p < 0.05$ ) amongst growing, resting and dead cells, ranging from 34 – 65 %, 29 – 56 % and 9 – 37 %, respectively. Remarkably, BL5022, BL5089, LA2404 and LA2410 strains were significantly more efficient in cholesterol removal than other probiotics ( $p < 0.05$ ) (Figure 4.3). Analysis of variance showed that the strain and the cell type significantly influenced cholesterol removal ability significantly.

The cholesterol removal ability of resting and dead cells of probiotics confirmed that their cellular membranes still had the ability to bind cholesterol. Accordingly, it is assumed

that even non-viable cells of probiotics could be used as cholesterol-reducing agents (Tok & Aslim, 2010). Similarly, it has been noted that resting and dead cells of *Lactobacillus* species are able to remove cholesterol (Kimoto et al., 2002; Liong & Shah, 2005). This implies that cholesterol removal is not only by the assimilation mechanism within growing probiotic cells, but also by an adhesion mechanism to cell membranes.

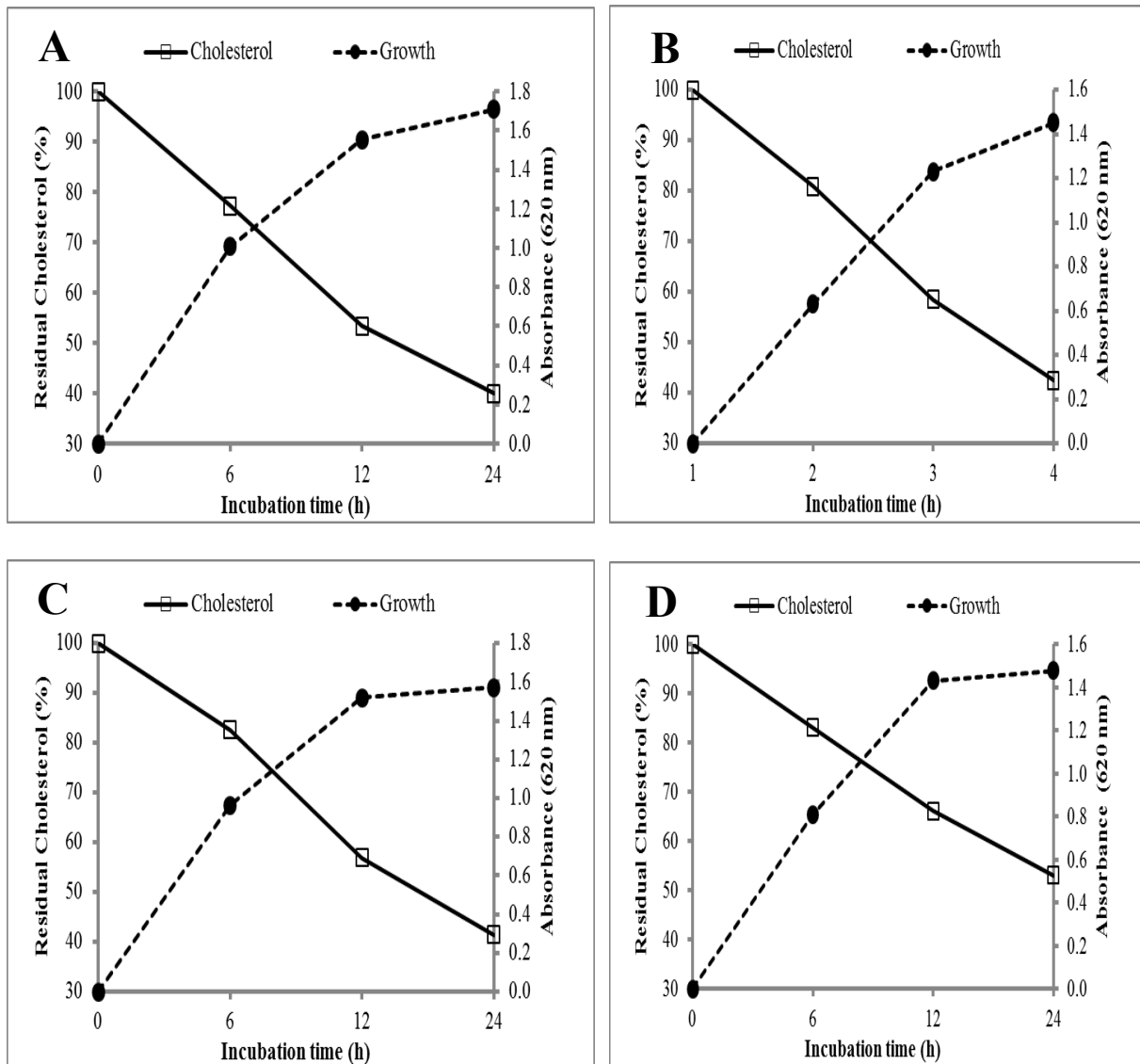
### **4.3.3 Deconjugation of Bile Salts**

#### *4.3.3.1 Free cholic acid*

The deconjugation activities of fourteen probiotics toward sodium glycocholate (glycine-conjugated salt), sodium taurocholate (taurine-conjugated salt) and their mixtures are shown in Figure 4.4. All probiotic strains were able to deconjugate both salts and their mixture to varying degrees. The amount of liberated cholic acid varied significantly ( $p < 0.05$ ) and ranged from 0.37 – 0.56 mM for sodium glycocholate, 0.07 - 0.40 mM for sodium taurocholate and 0.14 – 0.42 mM for their mixture. Overall, LA2410, LA2413, LPC279 and LZ15820 showed significantly higher bile salt deconjugation activities compared with other probiotics ( $p < 0.05$ ) (Figure 4.4). Fisher's test indicated significant differences ( $P < 0.05$ ) between probiotic bacteria in deconjugation of the same bile salt. All probiotics deconjugated sodium glycocholate to a higher degree ( $P < 0.05$ ) than taurocholate and their mixtures ( $p < 0.05$ ). LA2410, LZ15820, LR2607 and LPC279 liberated higher amounts of cholic acid from sodium glycocholate than the rest ( $P < 0.05$ ). Furthermore, most of the probiotics in this study resulted in more cholic acid from the mixture of bile salts, than sodium taurocholate salts except BL5089, BB5286 and LR1521.

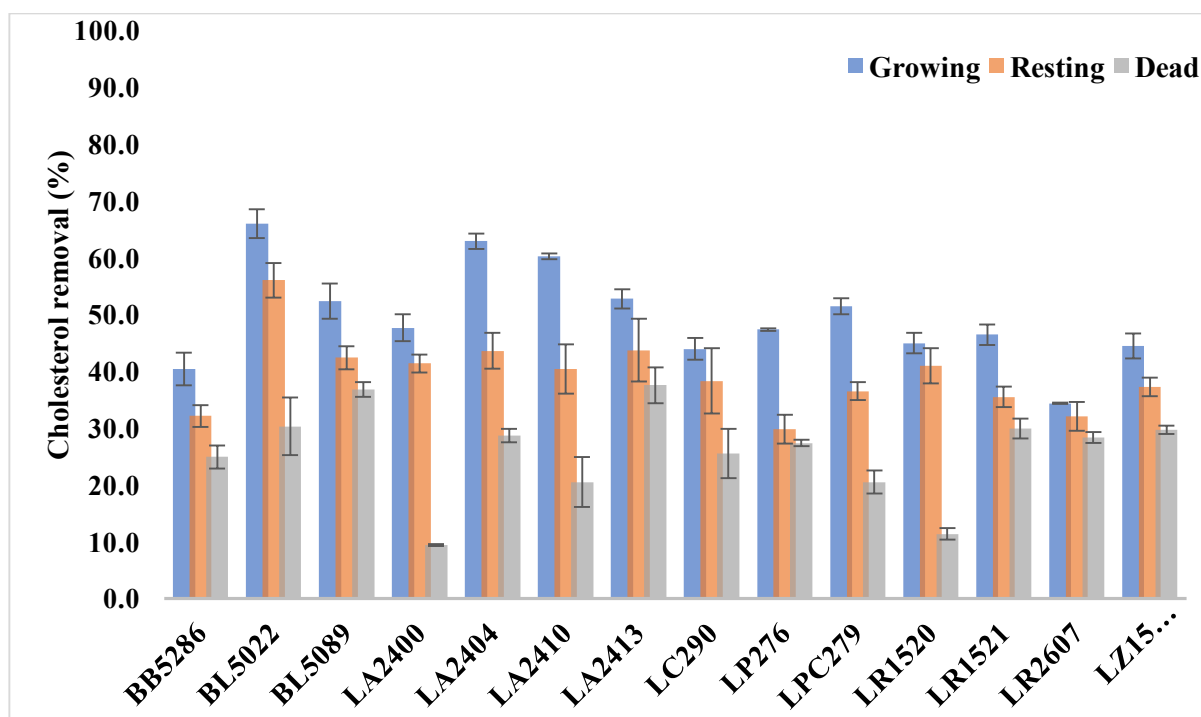
Conjugated bile salts including glycine-conjugated and taurine-conjugated salts play an important role in cholesterol absorption in the small intestine. As surfactants, conjugated bile salts contribute to cholesterol micelle formation, which enhances cholesterol absorption in the

small intestine (Araki et al., 1996). As a result of deconjugation of bile salts, the cholesterol micelle formation would be disturbed and thereby cholesterol absorption would be burdened (Cheeke, 2000). Klaver and Van der Meer (1993) reported a drop in the pH of the fermentation media due to natural acid production by culture and deconjugation of bile salts, cholesterol micelles destabilization and cholesterol co-precipitation with free bile acids.



**Figure 4.2:** Trend of residual cholesterol (%) and growth of BL5022 (A), LA2404 (B), LA2410 (C) and BB5286 (D) the incubated in MRS supplemented with 100  $\mu\text{g}$  /mL water-soluble cholesterol and 0.3 % oxgall for 24 h at 37  $^{\circ}\text{C}$ .

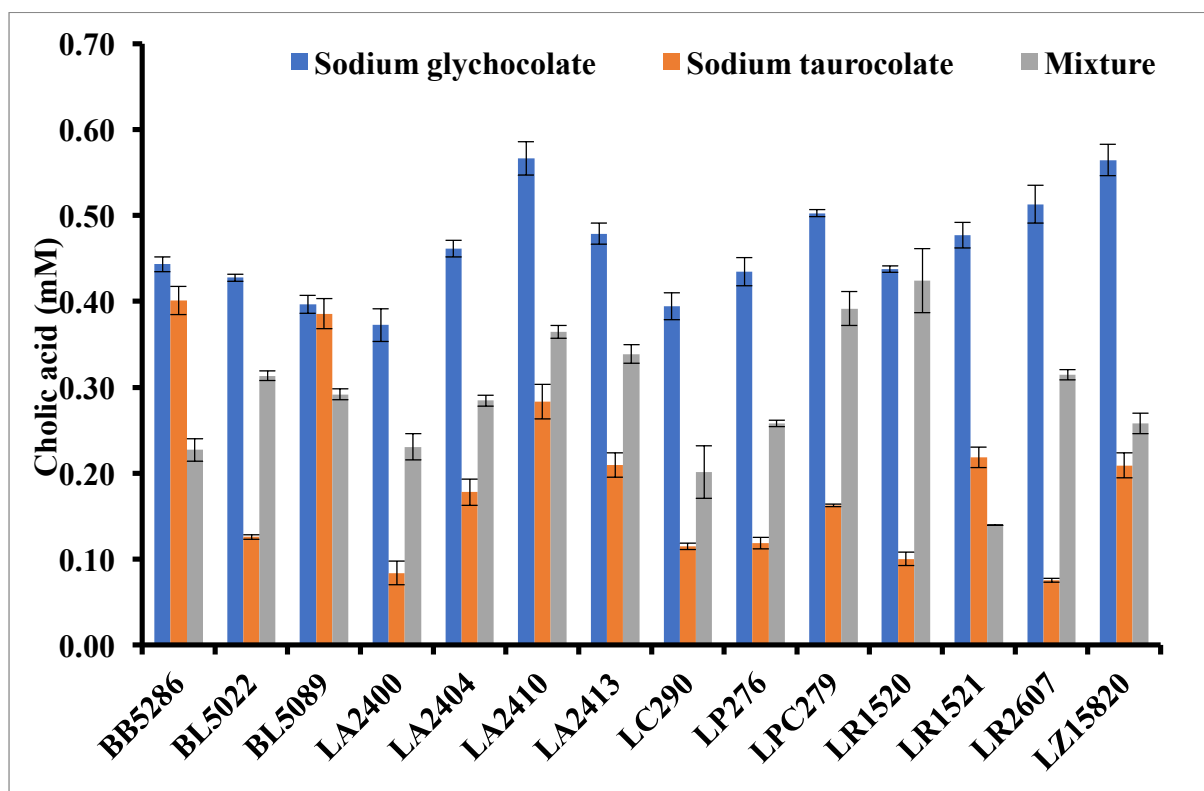
This study clearly demonstrates that all fourteen probiotic cultures deconjugated sodium glycocholate bile salt more efficiently than sodium taurocholate. These results are in agreement with those of others (Brashears et al., 1998; Ramasamy et al., 2010) where higher deconjugating activity resulted from *Lactobacillus* strains on sodium glycocholate-conjugates compared to sodium taurocholate-conjugates. Unlike glycine, taurine metabolism contributes to production of hydrogen sulphide, which is highly toxic to the host after deconjugation (Ridlon et al., 2010). Therefore, strains that are mostly able to deconjugate glycine but not taurine may be useful as probiotics. In this study, strains of *L. zeae* ASCC 15820, *L. acidophilus* CCCC 2410, *L. rhamnosus* ASCC 2607 and *L. plantarum* ASCC 279 showed higher deconjugating activity on sodium glycocholate than sodium taurocholate and the mixture of bile salts. Similarly, glycine conjugated bile salt is more efficiently deconjugated by strains of *L. acidophilus* from both human and porcine origins than taurine conjugated bile salt (Corzo & Gilliland, 1999). It is apparent that probiotic cultures may exhibit deconjugation activities as a defensive mechanism against the toxic effects of conjugated bile salts (Moser & Savage, 2001).



**Figure 4.3:** Cholesterol removal (%) of growing, resting and dead cells of the fourteen probiotic bacteria incubated in MRS supplemented with 100 µg/mL water-soluble cholesterol and 0.3 % oxgall for 24 h at 37 °C.

The concentration of individual bile salts used (6 mM) closely resembles that present in the human small intestine (Brashears et al., 1998; De Boever & Verstraete, 1999) and bile mixtures (2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate), which is similar to the amount in human bile (Lin et al., 1989). Herein, it is noted that all strains demonstrate greater deconjugation abilities when incubated with mixture of bile salts compared to sodium taurocholate alone which could be due to the lower concentrations (4 mM) of bile salt mixture (2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate) compared to a higher concentration of individual bile salts (6 mM). Higher bile salt concentrations may be suppressive to bacterial growth, inversely affecting their deconjugation activities. Since sodium glycocholate is the predominant component of bile salts in the human intestine, it was postulated that strains that prefer to deconjugate sodium glycocholate may be more effective

in lowering serum cholesterol concentrations (Brashears et al. (1998). Thus, with a high deconjugation activity of *L. zeae* ASCC 15820, *L. acidophilus* CSCC 2410, *L. rhamnosus* ASCC 2607, and *L. plantarum* ASCC 279 towards sodium glycocholate and sodium taurocholate at concentrations that resemble that of human bile, it is likely that these strains may exert strong *in vivo* deconjugation effects.



**Figure 4.4:** Bile deconjugation activities of the 14 probiotic strains incubated in MRS supplemented with sodium glycocholate (6 mM), sodium taurocholate (6 mM) and their mixture (4 mM) for 24 h at 37 °C.

**Table 4.1:** Bile salt hydrolysis (BSH) of 14 probiotics bacteria

Probiotic	Protein content mg/mL	Sodium glycocholate		Sodium taurocholate		Mixture	
		Total activity U/mL	Specific activity U/mg	Total activity U/mL	Specific activity U/mg	Total activity U/mL	Specific activity U/mg
BB5286	2.68±0.08 <sup>a</sup>	1.16±0.03 <sup>a</sup>	0.43±0.02 <sup>ab</sup>	0.76±0.08 <sup>cd</sup>	0.28±0.02 <sup>cd</sup>	1.20±0.35 <sup>bc</sup>	0.44±0.12 <sup>cde</sup>
BL5022	2.64±0.05 <sup>a</sup>	0.77±0.08 <sup>cd</sup>	0.29±0.03 <sup>cde</sup>	1.20±0.21 <sup>abc</sup>	0.45±0.07 <sup>abc</sup>	1.41±0.11 <sup>ab</sup>	0.53±0.03 <sup>abc</sup>
BL5089	2.81±0.08 <sup>a</sup>	0.71±0.08 <sup>cde</sup>	0.25±0.03 <sup>def</sup>	1.28±0.23 <sup>abc</sup>	0.46±0.09 <sup>abc</sup>	1.28±0.06 <sup>abc</sup>	0.45±0.01 <sup>bcde</sup>
LA2400	2.60±0.11 <sup>a</sup>	0.83±0.04 <sup>bc</sup>	0.32±0.01 <sup>cd</sup>	1.33±0.34 <sup>ab</sup>	0.52±0.13 <sup>ab</sup>	1.39±0.09 <sup>ab</sup>	0.53±0.02 <sup>abc</sup>
LA2404	2.67±0.05 <sup>a</sup>	0.54±0.04 <sup>fg</sup>	0.20±0.01 <sup>fgh</sup>	0.83±0.13 <sup>bcd</sup>	0.31±0.06 <sup>bcd</sup>	1.11±0.05 <sup>bc</sup>	0.42±0.01 <sup>cde</sup>
LA2410	2.72±0.13 <sup>a</sup>	0.57±0.05 <sup>efg</sup>	0.21±0.02 <sup>efgh</sup>	1.04±0.20 <sup>abcd</sup>	0.39±0.09 <sup>abcd</sup>	1.04±0.04 <sup>bc</sup>	0.39±0.03 <sup>cde</sup>
LA2413	2.69±0.04 <sup>a</sup>	0.46±0.08 <sup>hg</sup>	0.17±0.03 <sup>gh</sup>	0.60±0.04 <sup>d</sup>	0.22±0.01 <sup>d</sup>	0.92±0.14 <sup>c</sup>	0.34±0.06 <sup>de</sup>
LC290	2.59±0.09 <sup>a</sup>	0.97±0.03 <sup>b</sup>	0.37±0.02 <sup>bc</sup>	0.78±0.08 <sup>cd</sup>	0.30±0.02 <sup>cd</sup>	1.20±0.07 <sup>bc</sup>	0.46±0.02 <sup>bcde</sup>
LP276	2.66±0.13 <sup>a</sup>	0.34±0.00 <sup>h</sup>	0.13±0.00 <sup>h</sup>	0.58±0.01 <sup>d</sup>	0.22±0.01 <sup>d</sup>	0.88±0.10 <sup>c</sup>	0.33±0.03 <sup>e</sup>
LPC279	2.74±0.14 <sup>a</sup>	0.66±0.06 <sup>def</sup>	0.24±0.02 <sup>defg</sup>	1.03±0.15 <sup>abcd</sup>	0.38±0.08 <sup>abcd</sup>	1.64±0.26 <sup>a</sup>	0.61±0.12 <sup>ab</sup>
LR1520	2.60±0.20 <sup>a</sup>	1.19±0.09 <sup>a</sup>	0.47±0.07 <sup>a</sup>	1.39±0.16 <sup>a</sup>	0.53±0.02 <sup>a</sup>	1.63±0.10 <sup>a</sup>	0.64±0.08 <sup>a</sup>
LR1521	2.54±0.04 <sup>a</sup>	0.47±0.06 <sup>gh</sup>	0.18±0.02 <sup>fgh</sup>	1.14±0.33 <sup>abc</sup>	0.45±0.13 <sup>abc</sup>	1.28±0.01 <sup>abc</sup>	0.50±0.00 <sup>abcd</sup>
LR2607	2.61±0.13 <sup>a</sup>	0.63±0.06 <sup>def</sup>	0.24±0.03 <sup>defg</sup>	1.24±0.17 <sup>abc</sup>	0.47±0.06 <sup>abc</sup>	1.19±0.03 <sup>bc</sup>	0.46±0.03 <sup>bcde</sup>
LZ15820	2.76±0.10 <sup>a</sup>	0.52±0.03 <sup>fg</sup>	0.19±0.00 <sup>fgh</sup>	0.84±0.11 <sup>bcd</sup>	0.31±0.04 <sup>cd</sup>	1.11±0.15 <sup>bc</sup>	0.40±0.06 <sup>cde</sup>

<sup>a</sup> MRS broth supplemented with sodium glycocholate (6 mM), sodium taurocholate (6 mM), or mixture (6 mM) consisted of glycocholic acid, glycochenodeoxycholic acid, taurocholic acid, taurochenodeoxycholic acid, and taurodeoxycholic acid.

<sup>b</sup> Values are means of triplicates (Mean ± standard error)

<sup>c</sup> Different small letters within the same column indicate statistical significant ( $P \leq 0.05$ )



#### 4.3.3.2 Bile salt hydrolysis

The protein contents and the bile salt hydrolysis activities of the fourteen probiotic bacteria, expressed in total activity and specific activity are presented in Table 4.1. An analysis of variance indicated that the bacterial species had an effect on the hydrolysis of sodium glycocholate (glycine-conjugated salt), sodium taurocholate (taurine-conjugated salt) and their mixtures ( $P < 0.05$ ). The specific activity of BSH varied between each probiotic strain used and ranged from 0.13 – 0.47 U/mg (sodium glycocholate), 0.22 – 0.53 U/mg (sodium tarocholate), and 0.33 – 0.64 U/mg (mixture) ( $P < 0.05$ ) (Table 1.1). LA2400 showed higher specific BSH activity with all bile salts, and LR1520 the highest compared to all probiotics ( $P < 0.05$ ). Of note is, BL5022, BB5286 and BL5089 demonstrated moderate BSH specific activities with all bile salts, and, LA2413 and LP276 exhibited lower BSH specific activities with all bile salts ( $P < 0.05$ ).

The BSH activity has been reported as an indicator of cholesterol lowering by probiotics. The BSH enzyme produced by probiotics liberates amino acids from conjugated bile salts and produces free bile acids (Kimoto et al., 2002; Liong & Shah, 2005) that are more likely excreted with human faeces than reabsorbed in the human lumen (Center, 1993). Moreover, the decomposition of bile salts by the BSH enzyme would disrupt the formation of the cholesterol micelle, which in turn prevents cholesterol absorption (Klaver & Van der Meer, 1993). The higher BSH activity toward sodium taurocholate rather than sodium glycocholate observed in this study contradicts previous findings of Liong and Shah (2005) although different strains were used amongst these studies.

Results on the BSH activity showed that all strains, except *L. acidophilus* CSCC 2413 and *L. plantarum* ASCC 279 exhibited, higher BSH specific activity towards a mixture of glycine and taurine conjugated bile compared to the individual conjugated bile salts. The higher

BSH activity by most strains in the presence of bile salt mixture may be due to a lower concentration of bile mixture (4 mM) compared to a higher individual concentration of conjugated bile (6 mM). Therefore, when more cholic acid is liberated from individual conjugated bile salts (as compared to the mixture of conjugated salts), it may lead to bacteriototoxicity, growth inhibition, and a lower BSH specific activity from most strains studied. Further studies are required to ascertain the correlation between BSH activity and end product toxicity. The lower BSH activities of some probiotic strains used in this study may be due to low levels of BSH enzyme production or differences in enzyme structure. Tanaka et al. (2000) reported that the molecular weight and structure of BSH is strain dependent. On this basis, this study postulates that the changes observed in BSH activities may be due to differences in the putative active sites of the BSH enzymes.

#### **4.3.4 Fatty acids profile of cellular membrane**

The change in fatty acids profile of fourteen probiotics cellular membranes inoculated in MRS with or without cholesterol is shown in Table 4.2. Three saturated (myristic, C14; palmitic, C16; stearic, C18) and two unsaturated fatty acids (myristoleic, C16:1 and oleic, C18:1) were continuously analysed in all 14 probiotics as indicators to track the change in fatty acid composition of these strains in presence or absence of cholesterol. As expected, the presence of cholesterol in MRS significantly ( $P < 0.05$ ) affected the fatty acid composition of each probiotic strain (Table 4.2). Analysis of variance indicated that the percentage of fatty acids differed between strains in the presence or absence of cholesterol ( $P < 0.05$ ). The changes in the fatty acid composition may be attributed to cholesterol assimilation occurring during growth of probiotics in MRS with cholesterol. Of interest, the fatty acid profile of *Lactococcus lactis* cells grown with or without cholesterol differs significantly (Kimoto et al., 2002)

**Table 4.2** Fatty acid compositions of fourteen probiotics cultured separately in MRS with or without cholesterol

		Fatty acids (%) <sup>a</sup>						
		C14	C16	C16:1	C18	C18:1	SFA <sup>b</sup>	UFA <sup>c</sup>
<b>LR1521</b>	without cholesterol	36.16±1.16	37.45±1.05	10.46±0.02	6.91±0.28	9.03±0.19	80.51±0.17	19.49±0.17
	with cholesterol	18.57±0.50	7.89±0.01	9.99±0.40	31.14±1.32	32.41±0.43	57.60±0.83	42.40±0.83
<b>LA2400</b>	without cholesterol	34.40±0.73	36.62±0.04	15.36±0.81	7.53±0.03	6.09±0.01	78.55±0.80	21.45±0.80
	with cholesterol	16.41±0.54	38.87±2.00	9.86±0.37	14.00±0.39	20.87±0.69	69.27±1.06	30.73±1.06
<b>LR2607</b>	without cholesterol	15.79±0.06	47.80±0.73	20.95±0.96	9.41±0.10	6.05±0.07	73.00±0.89	27.00±0.89
	with cholesterol	25.73±0.55	38.14±0.44	11.91±0.11	3.92±0.03	20.29±1.12	67.79±1.01	32.21±1.01
<b>LR1520</b>	without cholesterol	49.47±0.13	22.02±0.08	15.97±0.25	3.41±0.02	9.12±0.03	74.90±0.22	25.10±0.22
	with cholesterol	17.42±0.09	49.91±0.05	7.48±0.07	4.23±0.00	20.96±0.06	71.56±0.14	28.44±0.14
<b>LPC279</b>	without cholesterol	31.00±0.15	30.62±0.15	27.66±0.02	6.51±0.05	4.21±0.04	68.13±0.06	31.87±0.06
	with Cholesterol	31.21±0.26	37.80±0.04	17.87±0.08	4.89±0.08	8.23±0.06	73.90±0.14	26.10±0.14
<b>LZ15820</b>	without Cholesterol	43.73±1.37	32.34±0.73	14.51±0.43	6.28±0.16	3.13±0.04	82.36±0.48	17.64±0.48
	with Cholesterol	26.93±0.04	47.01±0.11	14.06±0.02	3.63±0.01	8.37±0.13	77.56±0.15	22.44±0.15
<b>LC290</b>	without Cholesterol	25.13±0.82	45.35±0.46	7.79±0.17	8.32±0.10	13.41±0.10	78.80±0.26	21.20±0.26
	with Cholesterol	20.66±0.13	44.77±0.10	14.01±0.02	5.97±0.07	14.58±0.11	71.41±0.09	28.59±0.09
<b>BB5286</b>	without Cholesterol	26.45±0.32	45.96±0.18	12.48±0.50	9.75±0.04	5.36±0.04	82.17±0.47	17.83±0.47
	with Cholesterol	24.88±0.08	19.87±0.58	14.38±0.59	7.78±0.23	33.10±0.17	52.52±0.42	47.48±0.42
<b>LA2404</b>	without Cholesterol	25.36±0.06	42.44±0.04	18.82±0.01	7.43±0.02	5.95±0.05	75.23±0.04	24.77±0.04
	with Cholesterol	32.80±0.46	35.02±0.23	10.01±0.18	4.10±0.02	18.07±0.85	71.92±0.67	28.08±0.67
<b>LA2410</b>	without Cholesterol	17.29±0.03	43.96±0.14	26.96±0.20	6.83±0.03	4.96±0.06	68.08±0.14	31.92±0.14
	with Cholesterol	29.24±0.10	28.40±0.22	8.27±0.02	3.15±0.06	30.94±0.04	60.79±0.06	39.21±0.06
<b>LA2413</b>	without Cholesterol	30.16±0.30	42.37±0.33	12.93±0.75	8.61±0.08	5.93±0.05	81.14±0.70	18.86±0.70
	with Cholesterol	30.10±0.30	35.22±0.63	8.45±0.16	5.48±0.17	20.75±1.26	70.81±1.10	29.19±1.10
<b>BL5022</b>	without Cholesterol	23.44±1.08	22.07±0.18	21.92±0.52	3.62±0.03	28.96±0.34	49.12±0.86	50.88±0.86
	with Cholesterol	25.77±0.28	31.70±0.39	19.47±0.37	13.90±1.02	9.15±0.02	71.38±0.35	28.62±0.35
<b>BL5089</b>	without Cholesterol	25.97±0.01	40.67±0.02	22.45±0.03	5.23±0.02	5.68±0.02	71.87±0.01	28.13±0.01
	with Cholesterol	28.13±0.03	37.98±0.01	19.31±0.09	5.59±0.03	8.99±0.04	71.70±0.05	28.30±0.05
<b>LP276</b>	without Cholesterol	20.29±0.27	40.42±1.14	15.45±0.52	4.94±0.12	18.90±1.01	65.65±1.53	34.35±1.53
	with Cholesterol	50.53±1.00	23.64±1.16	12.58±0.15	3.54±0.00	9.71±0.01	77.71±0.17	22.29±0.17

<sup>a</sup> C14 = Mistyric acid; C16 = Palmitic acid; C18 = Stearic acid; C16:1 = Myristoleic acid; C18:1 = Oleic acid ; <sup>b</sup> SFA: Saturated fatty acids <sup>c</sup> UFA: Unsaturated fatty acids Values : mean ± SEM (n=6)

Furthermore, the resistance of *L. acidophilus* ATCC 43121 cells toward sonication is greater when they grow in the presence of cholesterol (Noh et al., 1997); suggesting that cholesterol in the media may be incorporated into the cell or attached to cellular membranes, which in turn alter the fatty acid profile of the membranes of these cells.

The changes in the fatty acid profile of the cellular membrane in bacteria show evidence for cholesterol incorporation into the cellular membrane, and, indeed, using fluorescence anisotropy, cholesterol is noted in cellular membranes (Lye et al., 2010).

The initial objective of this study was to select strains of probiotics with maximum cholesterol removal ability through *in vitro* assays of fourteen strains. Four strains were selected and were further assayed for acid and bile salts tolerance and antihypertensive properties (data not shown) and two strains were identified as the most suitable, *L. acidophilus* ASCC 2404 and *L. rhamnosus* ASCC 1520 for application in the formulation of a synbiotic yogurt. The resulting functional product is currently being used in human trials with hypercholesterolemic and hypertensive subjects to further document their health benefits. The findings of this part of the study will be reported at a later stage.

#### **4.4 CONCLUSIONS**

The current study suggests that all probiotic strains studied possessed varying degrees of cholesterol removal capabilities from the media through several mechanisms, including cholesterol assimilation, incorporation of cholesterol into cellular membrane, binding of cholesterol to cells and bile salt deconjugation. However, the cholesterol incorporation and binding to cell membrane mechanisms of probiotics are the most likely mechanisms compared to other studied mechanisms. In particular, BL5022, LA2404, LA2410 and BB5286 demonstrated higher cholesterol removal ability compared to other strains. The cholesterol assimilation by growing cells of probiotics was significantly higher than resting and dead cells.

However, cholesterol removal by resting and dead cells confirmed that even non-viable probiotics still had the ability to bind to cholesterol, and therefore, could be used as cholesterol-reducing agents in the gastrointestinal system. All *Lactobacilli* and *Bifidobacteria* strains tested were able to deconjugate both sodium glycocholate and sodium taurocholate. Strains of LZ 15820, LA 2410, LR 2607, and LP 279 showed higher deconjugating activity in the presence of sodium glycocholate than sodium taurocholate and the mixture of bile salts. Substrate preference for bile salt hydrolysis was more towards sodium glycocholate than sodium taurocholate. LA2400 and BL5022 exhibited higher bile salt hydrolysis specific activity towards all bile salts. This study confirmed the suitability of BL5022, LA2404, and LA2410 for application in functional foods, especially where cholesterol reduction in food is the main aim. Based on these findings and the results of gastric acidity resistance, intestinal salts tolerance and antihypertensive properties of LA 2404 and LR 1520 (data not shown), we have developed a functional synbiotic yogurt has been developed that is currently being used in human trials to further document its health benefits.

## **5. DEVELOPMENT OF SYNBIOTIC YOGURT SUPPLEMENTED WITH POMEGRANATE JUICE**

## 5.1 INTRODUCTION

In the current food market, dairy products such as yogurt, fermented milks and cheese dominate the probiotic food sector (Madhu et al., 2012). In the past three decades, the worldwide production of various fermented milk products has increased by more than 50 percent, from 500 million tonnes in 1983 to 769 million tonnes in 2013 (FAO, 2017), with yogurt as a major contributor due to its popularity stemming from its high nutritional value and desirable health effects. For example, in Australia, yogurt consumption has increased steadily from 5.6 kg per capita in 2001 to 7.2 kg in 2015 (Dairy Australia, 2016). The minimum daily consumption of the probiotic yogurt to deliver its positive bioactive effects is suggested at 100 g containing over  $10^6$  CFU g<sup>-1</sup> (Madhu et al., 2012).

Continued efforts are being made to improve the health status of humans by modulating the intestinal microbiota using live microbial adjuncts, namely probiotics. Probiotic organisms require a vehicle to reach the site of action; the gastrointestinal tract (GIT) of the human body. The vehicle is generally a food product, which contains these live bacteria. The products should have a lengthy shelf-life with a minimum cell count of more than  $10^6$  CFU g<sup>-1</sup> at the point of consumption. The product goes through the harsh conditions of gastric acid and bile salts before reaching the colon, therefore the probiotics should be able to survive this harsh transition through the GIT (refer to section 3.3.2 and 3.3.3). Scientific evidence suggests that probiotic bacteria consumed at a level of  $10^9$ - $10^{11}$  CFU g<sup>-1</sup> per day can decrease the incidence and severity of some intestinal disorders (Zubillaga et al., 2001).

The lactic acid bacteria (LAB) used as yogurt starter cultures e.g. *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* do not tolerate the gastric acidity or intestinal bile, and those who survive these harsh conditions are unable to attach to the intestinal epithelium, or grow in the lower intestinal tract to be able to provide any health benefits (Fooks & Gibson, 2002). Therefore, in order to maintain the intestinal microflora balance, the intake of probiotic strains

as a preventive approach is recommended. However, these strains must be resistant enough to survive the harsh conditions present in the GIT and compete with the resident microflora. The possible mechanisms of probiotic effect in the control of gastrointestinal health include 1) the excretion of acids (e.g. lactate and acetate), 2) competition for nutrients and gut receptor sites, 3) immune-modulation, and 4) production of specific anti-microbial agents (Erickson & Hubbard, 2000). In addition, it has been suggested that some probiotics can help to maintain the remission phase in inflammatory conditions such as ulcerative colitis and irritable bowel syndrome (Aragon et al., 2010).

The approach of modulating the gut flora for improved health has much relevance for the management of acute and chronic gut disorders. However, due to the slow growth rate of probiotic and survival issues associated with transit time and establishment in the competitive gut ecosystem, the use of prebiotics in association with useful probiotics known as ‘synbiotics’ may allow a more efficient implantation of probiotic bacteria in the colon. This is because prebiotics have a stimulating effect on the growth and activities of exogenous and endogenous bacteria. In the past two decades, there has been a significant increase in the popularity of synbiotic yogurt with the combination of some LAB including *L. acidophilus*, *L. casei*, *B. animalis* ssp. and inulin-type fructans (Roberfroid, 2007; Paseephol & Sherkat, 2009; Ramachandran & Shah, 2010; Sheu et al., 2010).

To select the most efficient probiotic strain to be used in the development of our synbiotic yogurt, eleven strains of *Lactobacilli* and three strains of *Bifidobacteria* provided by Dairy Innovation Australia Limited (Werribee, VIC, Australia) were evaluated for their acid and bile tolerance, antihypertensive properties and cholesterol removal ability (Section 3.2.1). Among these strains, *L. rhamnosus* ASCC 1520 and *L. acidophilus* CSCC 2404 were chosen as they possess reasonable acid and bile tolerance, and good hypotensive and cholesterol removal properties (Sections 3.3 and 4.3.1).



In addition to synbiotics, there has been an increased interest in dietary sources of antioxidant polyphenols in red fruit juices such as pomegranate (*Punica granatum L.*) due to their strong antioxidant activity (AA) resulting from high bioactive compounds such as phenolics, flavonoids, ellagitannins (ETs), and proanthocyanidin compounds, mineral and complex carbohydrates. A single serving of pomegranate juice (PJ) (240 mL), provides 65% of the recommended daily intake (RDI) for vitamin B6 and riboflavin, 4% RDI for biotin, niacin and pantothenic acid, and 2% RDI for thiamine. Potassium is the most abundant mineral in PJ, with a single serving providing 520 mg per day or 15% RDI. It also provides 2% RDI calcium, iron, zinc, and phosphate (McCutcheon et al., 2008).

To establish the scientific evidence of the health-promoting properties of probiotics and polyphenols, a dairy-based functional food containing probiotic bacteria was developed. Physicochemical and functional properties of the developed product and the effects of PJC supplementation on antioxidant and antihypertensive activities and hypocholesterolemic properties of the yogurt during the refrigerated storage for twenty-one days were analysed.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Source and Activation of the Cultures**

The selected probiotic strains *L. rhamnosus* 1520 and *L. acidophilus* 2404 were used for the preparation of synbiotic yogurt. The stock cultures were then stored at -80°C in MRS broth supplemented with 40 % (v/v) glycerol as a cryoprotectant. Prior to use, the frozen cultures were first thawed at room temperature inside the laminar flow cabinet for 15–20 min, then activated by triplicate subculturing at 1% (v/v) level in sterile MRS broth (Oxoid, SA. Australia) under an anaerobic condition at 37°C overnight. The activated cultures were further inoculated (1% v/v) into 10 mL aliquots of sterilized reconstituted skim milk (RSM)

supplemented with glucose (2g/100 mL) and incubated for 4–6 h at 37°C before inoculation into milk.

## **5.2.2 Yogurt Production**

### *5.2.2.1 Control Yogurt*

The control set yogurt (coded C) was prepared according to Kusuma et al. (2009). Briefly, the low-heat skim milk powder (LHSMP, 0.9% milk fat, 34% protein) (Bonlac Foods Limited, Melbourne, Victoria, Australia) was reconstituted with distilled water to 16% solid content, then heat-treated at 90 °C for 10 min, cooled to 43 °C and aseptically inoculated with 0.02% (w/v) of freeze-dried Yoflex 380 culture (a 50:50 mixture of *S. thermophilus* and *L. bulgaricus*), as direct vat inoculation, recommended by the supplier (Chr. Hansen Pty. Ltd., Bayswater, Vic., Australia). After a gentle stirring for 10-15 min to distribute the culture evenly, the inoculated milk samples were aseptically transferred into 100 mL plastic containers, tightly sealed and incubated at 43 °C. Upon reaching pH 4.7, the samples were transferred to a cold room at 4 °C and kept for three weeks.

Despite the fact that all these yogurt samples were served on the first week post production to the human subjects, the microbiological and physicochemical evaluations were conducted for three weeks post manufacturing.

### *5.2.2.2 Synbiotic Yogurt*

The yogurt samples were manufactured as per section 5.2.2.1 and then, four commercially available prebiotics ingredients, i.e. D-Sorbitol [98%, Sigma-Aldrich), D-Mannitol (99%, Sigma-Aldrich), fructooligosaccharides (FOS) from chicory root ( $\geq 90\%$  FOS, Sigma-Aldrich) and Synergy 1 [fructooligosaccharides-enriched inulin (FEI); Invita Australia Pty. Ltd., Wheelers Hills, Vic., Australia] were compared to select the most appropriate type and concentration of prebiotic compound for synbiotic yogurt development.

The synbiotic yogurt (coded Syn) was also prepared following Kusuma et al. (2009) protocol. Allowing for 2% prebiotic ingredient, the solid content of the RSM was adjusted to 14% then heat-treated at 90 °C for 10 min, cooled to 43 °C and inoculated with 1) Yoflex 380 at 0.02% (w/v) level and, 2) the activated probiotic cultures *L. rhamnosus* 1520 (7.92 Log<sub>10</sub> CFU mL<sup>-1</sup>) and *L. acidophilus* 2404 (7.87 Log<sub>10</sub> CFU mL<sup>-1</sup>), each at 1% (v/v) level. After a slow agitation for 10-15min to distribute the cultures evenly, the milk was aseptically divided into four lots and 1% (w/v) of one type of the selected prebiotics was added to each lot and thoroughly mixed for complete distribution of the prebiotic. The incubation, cooling and storage of yogurt samples were as described above. Samples were drawn for evaluation at weekly intervals up to the third week. In addition, this trial did not need any control yogurt as the growth promotion properties of probiotics were compared among the samples.

The pH and texture profile of all yogurt samples as well as the probiotic viability of synbiotic yogurt samples were determined (Madhu et al., 2012) and the sample that took the least time to arrive to the target pH of 4.7, had the best texture profile and maximum probiotic viability was chosen for product optimisation trials by varying the prebiotic concentration between 1.0, 1.5 and 2.0 % (w/v).

#### 5.2.2.3 *Synbiotic Yogurt Supplemented with of Pomegranate Juice Concentrate (PJC)*

Based on previous studies in our research team (Arjmand, 2011) , among the commercially available PJ in the Australian market, the imported POM Wonderful PJ, made from the whole California-grown Wonderful pomegranate variety, was selected for this study. The single strength juice (14.5 ± 0.1°B) was concentrated to 52°B using a climbing film evaporator (Jobling, James A Jobling and Co Ltd, Stock-on-Trent, UK) (Appendix 5.1), and added to the inoculated milk at different concentrations 10, 12.5, 15 and 20% (w/v)] either before or after heat treatment. The control yogurt contained no PJC. To increase the supplementation level of

PJC, its initial pH ( $3.02 \pm 0.01$ ) was adjusted with food grade NaOH (7N) to 5 before adding to milk. PJC\* stands for pH-adjusted PJC. Above 20% PJC supplementation, the milk curdled so it was necessary to adjust its pH to 6.5 post PJC supplementation. To compensate for the dilution effect of PJC\* addition, the protein content of these samples was kept constant by adjusting the amount of LHSMP (14%) added in their formulations (Table 5.1).

**Table 5.1** Yogurt production protocol

Code	Base	Type of supplement	Ingredients (%)			Culture type
			LHSMP <sup>1</sup>	Distilled water	Percent of supplementation	
SynP	RSM	PJC* <sup>2</sup> Prebiotic	14	64	20 2	Yoflex 380 <i>L. rhamnosus</i> 1520 <i>L. acidophilus</i> 2404
Syn	RSM	Prebiotic	14	84	2	Yoflex 380 <i>L. rhamnosus</i> 1520 <i>L. acidophilus</i> 2404
C	RSM	-	16	84	-	Yoflex 380

<sup>1</sup> LHSMP: Low heat skim milk powder (TS: 96%, Pr: 36.1 %)

<sup>2</sup> PJC\*: pH-adjusted PJC

The prebiotic type and amount chosen for synbiotic yogurts supplemented with PJC (coded Synp) was determined based on the trials conducted in section 5.2.2.2 above. The production protocol was as described above. The procedure for yogurt manufacturing (C, Syn, and Synp) is shown in Appendix 5.2.

### 5.2.3 Microbiological Analyses

#### 5.2.3.1 The effect of selected prebiotics on probiotic growth during storage

The growth rate of probiotic bacteria (*L. rhamnosus* ASCC 1520 and *L. acidophilus* CSCC 2404) in synbiotic yogurts with or without PJC supplementation was evaluated during incubation and 21-day storage period.

### 5.2.3.2 *The effect of different concentrations of PJC on probiotic growth during storage*

The effect of PJC on the growth and viability of yogurt cultures and selected probiotic strains was assessed over the 21 days of storage period at 4°C in supplemented and control batches.

The sampling schedule for testing was 24 h post-incubation (day 1) and at weekly intervals for three weeks. The viability of each strain in different samples was calculated using the following equation adapted from Paseephol and Sherkat (2009):

$$\% \text{ Viability} = (\text{CFU mL}^{-1} \text{ after storage for 21 days} / \text{CFU mL}^{-1} \text{ on day 1}) \times 100$$

### 5.2.3.3 *Enumeration of bacteria*

Ten-gram samples of each yogurt was diluted with 90 mL of 0.15% sterile peptone water. Ten-fold serial dilutions ( $10^{-2}$  to  $10^{-8}$ ) were prepared in 9 mL of 0.15% sterile peptone water. Based on preliminary studies, 1 mL of the last four dilutions were used in duplicate for enumeration (10% vol/vol) of *L. rhamnosus* ASCC 1520 and *L. acidophilus* CSCC 2404 into MRS broth using pour plate technique. All plates were gently mixed clockwise and anticlockwise to disturb the samples uniformly, then allowed to set. Duplicate plates were incubated under anaerobic condition (using Gas-pack system, AnaeroGen-1.3) at 37 °C for 72 h. The numbers of Colony Forming Units (CFU) on plates containing 15 to 300 colonies (AS 5013.5-2004) were calculated per gram of sample as below:

$$\text{CFU g}^{-1} = (\text{Number of colonies} \times \text{Volume of dilute suspension}) / \text{Dilution factor}$$

## 5.2.4 **Physicochemical analyses**

### 5.2.4.1 *Soluble and total solids measurement*

The refractive index of fresh PJ and PJC samples was determined in triplicate according to AOAC method 932.12 (AOAC, 2002) with a calibrated Shibuya hand-held refractometer (Shibuya Optical Co., Ltd., Saitama Prefecture, Japan) and reported as degree Brix (°B). Total

solids of milk and yogurt samples were determined using an oven method according to Australian Standard (AS 2300.1.1-2008).

#### 5.2.4.2 *pH value*

The pH values of all yogurt samples were measured in triplicate using a pH-meter (HI 8424, Hanna instruments, Ann Arbor, MI, USA) previously calibrated with pH 7.0 and 4.0 standard buffers at 17-20 °C. The pH of milk and yogurt samples were determined according to Australian Standards (AS 2300.1.6-2010). The pH values of yogurt samples were measured on a weekly basis during storage.

#### 5.2.4.3 *Determination of cholesterol removal ability*

Thirty milligrams of water-soluble cholesterol (polyoxyethanyl-cholesterol sebacate) (Sigma, St. Louis, MO, USA) was dissolved in 10 mL Milli-Q water and filter-sterilized using 0.45-µm filter (Millipore, Corp., Bedford, MA, USA) to obtain a stock solution of cholesterol. One hundred µL of the cholesterol stock solution was added to 1 mL of each yogurt sample and incubated at 37°C for 24 h and then samples were drawn for evaluation at weekly intervals up to the third week of storage. Each sample was centrifugated at  $4000 \times g$  for 20 min at 4°C. The cholesterol content of the supernatant was determined using a modified colorimetric method as described by Rudel and Morris (1973) with minor modifications detailed by Liong and Shah (2005). Briefly, 1 mL of the supernatant was mixed with 1 mL KOH 33% (w/v) and 2 mL 96% ethanol. The mixture was vortexed for 1 min followed by incubation at 37°C for 15 min, and then cooled to room temperature. Upon cooling, 2 mL of Milli-Q water and 3 mL of hexane were added to the mixture followed by vortexing for 1 min. The mixture was then allowed to settle until two layers were separated. The upper hexane layer was collected and evaporated under nitrogen gas. Two millilitres of o-Phthalaldehyde reagent (50 mg OPA dissolved in 100 mL glacial acetic acid; Sigma) were added and vortexed for 1 min to dissolve the residues. To

this, 0.5 mL of sulphuric acid (98%; Sigma) was added and vortexed for 1 min followed by resting for 10 min at room temperature before measuring the absorbance at 550 nm using UV-spectrophotometer (Pharmacia Novaspec II, Cambridge, England). The cholesterol concentration was read off a standard curve prepared using the cholesterol stock solution. All experiments were conducted in triplicate and assayed twice (n = 6). The ability of probiotics in each yogurt sample to assimilate cholesterol was expressed as the percentage of cholesterol removed at each week of storage period as follows:

$$\% \text{ of cholesterol removed} = \frac{100 - \text{residual cholesterol at each week of storage period}}{100} \times 100$$

#### 5.2.4.4 *Determination of antihypertensive activity*

The antihypertensive activity (ACE inhibition activity) of yogurt samples was measured according to Wanasundara et al. (2002) using hippuryl-histidyl-leucine (HHL) as the substrate through high performance liquid chromatography (HPLC). The HPLC standards, ACE enzymes (from rabbit lung) and hippuric acid (HA) were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). The assay was conducted in a Tris buffer (50 mM, pH 8.3) containing 300 mM NaCl. The assay sample consisted of 50 µL of 3.0 mM HHL, 50 µL of the ACE enzyme solution and 50 µL of each yogurt sample was placed in a glass tube, incubated for 30 min at 37°C in a water bath without mixing, and then vortexed for 1 min followed by incubation for an additional 30 min after mixing. Glacial acetic acid (150 µL) was added to stop the reaction. The sample thus prepared was kept at -20°C until analysis by HPLC. An aliquot (20 µL) of the test sample was injected onto the HPLC column consisting of a Varian 9012 solvent delivery system, a Varian 9100 auto sampler, a Varian 9050 variable wavelength UV-visible absorbance detector, and 730 data modules. The system was fitted with reverse-phase column (C18, 250 mm × 4.6 mm, 5 µm; Grace Vydac) with a guard column (10 mm, 12 mm, Grace Vydac). The separation was conducted at room temperature (~22°C) at a flow rate

of 0.8 mL/min. The subsequent mobile phase used an isocratic system consisting of 12.5% (v/v) acetonitrile (Merck) in MilliQ water, and the pH was adjusted to 3.0 using glacial acetic acid. The detection device was an UV-visible detector set at 228 nm. The control reaction mixture contained 50  $\mu$ L of buffer instead of the assay sample and was expected to liberate the maximum amount of HA from the substrate due to uninhibited ACE activity.

The amount of HA released from HHL by ACE in each yogurt sample was determined using the standard curve prepared for HA. The percentage inhibition of enzyme activity was calculated as follows:

$$\% \text{ Inhibition} = \frac{HA_C - HA_S}{HA_C} \times 100$$

Where  $HA_C$  and  $HA_S$  represent the amount of HA in the control and in the sample respectively.

#### 5.2.4.5 Determination of total phenolic compounds (TPC)

The method of Zheng and Wang (2001) was used for the determination of TPC in yogurt samples using Folin-Ciocalteu reagent (FCR) and gallic acid as standard, which is based on chemical reduction of a mixture of tungsten and molybdenum oxides. This method relies on the transfer of electrons in an alkaline medium from phenolic compounds to a mixture of phosphomolybdic and phosphotungstic acids to form blue complexes readable by a spectrophotometer (Ainsworth & Gillespie, 2007).

The yogurt sample (0.1 mL) was mixed with 0.9 mL of distilled water and incubated for 2h at room temperature ( $20 \pm 2^\circ\text{C}$ ) in a shaking water bath. To this, 1 mL of FCR reagent (1:2 dilution) and 2 mL of 10 %  $\text{Na}_2\text{CO}_3$  solutions were added. The mixture was centrifuged at  $20,000 \times g$  for 20 min, and the supernatant was decanted and filtered through Whatman No. 1 filter paper. The absorbance of the clear supernatant solution was measured at 765 nm. All experiments were out carried in triplicates. Results were expressed as milligrams gallic acid equivalent (GAE) in 100 mL yogurt sample.



#### 5.2.4.6 *Determination of antioxidant activity*

The antioxidant activity of the yogurt sample was determined as the ability of the yogurt sample to scavenge 1, 1- diphenyl-2-picrylhydrazyl (DPPH) radicals. A 0.1 mM DPPH radical was added to 1 mL of 95% ethanol solution. An aliquot of 0.8 mL of ethanolic DPPH solution was mixed with 0.2 mL of yogurt sample or 95 % ethanol (as control), vortexed well and incubated for 30 min at room temperature ( $20 \pm 2^{\circ}\text{C}$ ). The samples were centrifuged at  $13,000 \times g$  for 15 min at  $4^{\circ}\text{C}$  and the absorbance of the supernatant was measured spectrophotometrically at 517 nm. The antioxidant activity was expressed as follows:

$$\% \text{ DPPH scavenging} = (\text{sample absorbance} - \text{control absorbance}) / (\text{control absorbance}) \times 100$$

#### 5.2.4.7 *Determination of proteolytic activity*

The extent of proteolysis in the yogurt samples was determined by measuring the liberated amino acids and peptides using the o-phthaldialdehyde (OPA) method of Leclerc et al. (2002) with some modifications. The yogurt gel was then broken down by vortexing and a sample of 2.5 mL was taken and mixed with 5 mL of 0.75 % trichloroacetic acid, and filtered through Whatman No. 1 filter paper. The OPA reagent was prepared by adding 50 mg of OPA in 100 mL of glacial acetic acid and then three millilitres of OPA reagent was added to 150  $\mu\text{L}$  the permeate and mixed. The absorbance of the solution was measured using a UV spectrophotometer (Pharmacia Novaspec II, Cambridge, England) at 340 nm after 2 min at room temperature ( $20 \pm 2^{\circ}\text{C}$ ). The proteolytic activity of the samples was an indication of liberated amino acids and expressed as a difference in absorbance between different yogurt samples and the control.

#### 5.2.4.8 *Colour measurement*

The colour values of all yogurt samples were measured using a Chroma Meter CR-400 (Konica Minolta, Sensing, INC, Tokyo, Japan) according to the method described by Shwartz

et al. (2009) on day 1. The readings were expressed in dimensions of  $L^*$ ,  $a^*$ ,  $b^*$ ,  $C$  and  $H^\circ$ , where  $L^*$  indicates the luminosity of the sample colour. Values of  $a^*$  - to  $a^*$  + show colour change from green to red, while  $b^*$  - to  $b^*$  + expresses the colour range from blue to yellow. The chroma value ( $C$ ) is calculated as  $C = (a^{*2} + b^{*2})^{1/2}$  and indicates the colour intensity or saturation. Hue angle is calculated from  $H^\circ = \tan^{-1} (b^*/a^*)$  and reflects the visual colour appearance. The colour index is calculated from  $(180 - H^\circ) / (L^* + C)$  (Solomon et al., 2006; Schwartz et al., 2009). The mean values of triplicate readings were reported for each sample.

#### 5.2.4.9 Texture analysis

Gel firmness of set yogurt samples was determined according to Paseephol et al. (2008) and (Arjmand, 2011) using a TA-XT2 Texture Analyser (Stable Micro System Ltd., Surry, UK) with 5 kg load cell, which was operated using the software package Texture Expert Exceed (Version 1.00). A single compression test was performed using a 20mm diameter aluminium cylindrical probe (P20) at a speed of  $1.0 \text{ mms}^{-1}$  (i.e. pre-test, test and post-test withdrawal speed). The penetration depth was set to 75% of the gel height and the force value was set at 0.1 N (i.e. automatic trigger 0.1 N). The penetration force was plotted against the compression time and the fracture force (N) was defined as the first significant inflexion point as the probe penetrated the gel (maximum force prior to breakage of the gel), while the firmness (N) was defined as the maximum force on compression force-time curve (i.e. force for 75% penetration). The cohesiveness ( $\text{Ns}^{-1}$ ) is defined as forces of internal bonds, which keep the product complete; the viscosity ( $\text{Pas}^{-1}$ ) is the force per unit area resisting uniform flow; and the adhesiveness ( $\text{Ns}^{-1}$ ) is defined as the degree to which food sample sticks to mouth surfaces or teeth.

#### *5.2.4.10 Sensory evaluation*

Yogurt samples were prepared in 100 mL cups and stored overnight at 4 °C before being used for organoleptic evaluation. Although, the preferred sample size for untrained sensory panel is 100, however, (n=25) of Victoria University students and staff was the maximum number of panelists registered in this study. The panel received 2 × 1h sensory training prior to the study and then, scored the yogurt samples for aroma, colour, appearance, gel thickness, gel firmness, flavour and overall acceptability on a 10-point hedonic scale. The scale comprises a series of ten verbal categories ranging from ‘dislike extremely’ to ‘like extremely’ and is described as such in various sensory texts (AS 2542.1.1-2005). A sample of the sensory questionnaire given to the panel is shown in Appendix 5.3.

The samples were served immediately after removing them from the fridge at 4 °C. Panelists were then asked to open the lid and evaluate aroma first and then colour and appearance by visual observation. After breaking down the yogurt gel with a spoon and gently mixing the samples, gel thickness was rated. Finally, after placing the yogurt in their mouths, the gel firmness, flavour and overall acceptability were evaluated.

This study was approved by Victoria University Human Research Ethics Committee (HRE 13-039). Prior to the trial, a full explanation concerning the purpose and methodology of the study was given to the participants and written informed consent was obtained from all participants (Appendix 5.4).

#### **5.2.5 Statistical Analysis**

All physicochemical tests were conducted in triplicate while microbial experiments were performed in duplicates and the mean values ± standard deviation (SD) were reported.

One-way analysis of variance (ANOVA) was used to test differences among three experimental yogurts and the significance was tested at  $P < 0.05$ . Pearson correlation was

measured at  $P < 0.05$  between all variables. Data were analysed using SAS software version 9.2 (SAS., 2008).

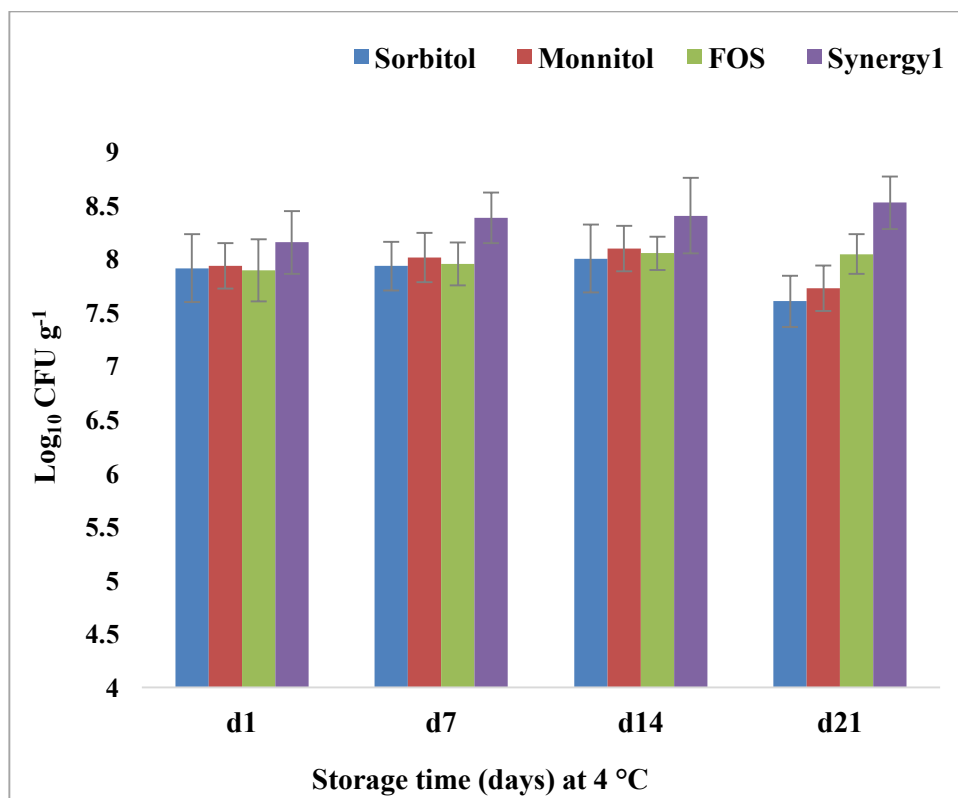
## **5.3 RESULTS AND DISCUSSION**

### **5.3.1 Selection of appropriate type of prebiotics for yogurt production**

To identify the best prebiotic for yogurt production, the effect of each selected prebiotic on the viable count of LAB, and the pH and texture profile of yogurt samples during the incubation and storage were examined.

#### *5.3.1.1 Viable count of LAB*

The viable count of LAB in the presence of the selected prebiotics during incubation and storage is presented in Figure 5.1. Although the cell count of LAB declined from  $7.91 \pm 0.31$  and  $7.93 \pm 0.21 \text{ Log}_{10} \text{ CFU g}^{-1}$  to  $7.6 \pm 0.23$  and  $7.72 \pm 0.21 \text{ Log}_{10} \text{ CFU g}^{-1}$  in yogurt supplemented with sorbitol and mannitol respectively, it was maintained at  $8.0 \pm 0.21 \text{ Log}_{10} \text{ CFU g}^{-1}$  throughout the 21-day storage period. Supplementation with Synergy 1 resulted in a significant ( $P < 0.05$ ) increase in the total count of LAB from  $8.15 \pm 0.29$  on day 1 to  $8.52 \pm 0.24 \text{ Log}_{10} \text{ CFU g}^{-1}$  on day 21. Thus, Synergy 1 was selected for the development of synbiotic yogurt. The results obtained were in agreement with Bartosch et al. (2005) and Meyer and Stasse-Wolthuis (2009), indicating that addition of Synergy 1 has been found to be more effective in producing beneficial health impacts and improved probiotic viability, rather than one prebiotic by itself due to the synergistic effect of the mixture of two prebiotic components (Refer to section 2.2.2).



**Figure 5.1** Effects of the selected prebiotics on total bacterial count ( $\log_{10}$  CFUg<sup>-1</sup>) in synbiotic yogurt during 21-day storage at 4°C.

#### 5.3.1.2 Changes in pH

Measurement of pH presents a critical quality control step in the production of dairy products, especially yogurt. The pH levels offers an indication of any bacterial or chemical contamination in a dairy product, as well as providing a convenient method to estimate the amount of acid development during processing and storage (Masulli, 2016). Post acidification can adversely affect the taste and smell and overall acceptability of yogurt, which often occurs during processing, storage and transportation and retail (Aryana & McGrew, 2007). In this study, the addition of 1% (w/v) of these selected prebiotics did not affect the fermentation time for the production of probiotic yogurts (data not shown). The changes in pH during refrigerated storage of yogurt samples supplemented with the selected prebiotics are shown in Table 5.2. The mean pH values ranged from  $4.10 \pm 0.05$  to  $4.70 \pm 0.03$  during storage period. There was a decline in pH with storage time and the yogurts supplemented with Synergy 1 and mannitol

had the highest and lowest pH values, respectively. The pH of mannitol was significantly ( $P < 0.05$ ) lower than the other treatments. This may be explained by mannitol having a shorter chain length than others, leading to a faster consumption by the probiotic bacteria, which results in more lactate and acetate production and therefore lower pH values (Aryana & McGrew, 2007). Synergy 1 could maintain the LAB viable counts at the higher level than necessary in order to characterise the developed probiotic yogurt as a synbiotic food, up to the twenty-first day of storage.

**Table 5.2** The effect of selected prebiotics on pH of yogurt samples during storage

Prebiotics Storage (days)	pH				
	Control	FOS	Synergy1	Sorbitol	Mannitol
<b>d1</b>	4.72± 0.01 <sup>aA</sup>	4.62 ± 0.01 <sup>aA</sup>	4.60 ±0.01 <sup>aA</sup>	4.70 ±0.06 <sup>aA</sup>	4.70±0.04 <sup>aA</sup>
<b>d7</b>	4.66± 0.02 <sup>aA</sup>	4.54 ± 0.06 <sup>aA</sup>	4.55 ±0.06 <sup>aA</sup>	4.62±0.03 <sup>aA</sup>	4.60±0.03 <sup>aA</sup>
<b>d14</b>	4.50 ±0.04 <sup>aB</sup>	4.40 ±0.06 <sup>aB</sup>	4.40 ±0.06 <sup>aB</sup>	4.35±0.05 <sup>aB</sup>	4.20±0.03 <sup>bB</sup>
<b>d21</b>	4.45 ±0.02 <sup>aB</sup>	4.25 ±0.06 <sup>aC</sup>	4.30 ±0.01 <sup>aC</sup>	4.20±0.03 <sup>aC</sup>	4.10±0.06 <sup>bC</sup>

Results presented as a mean (n = 3) ± SD.

Different lower- case letter superscripts depict the statistical difference within a row, ( $P < 0.05$ ) between means for different yogurt batches. Different capital letter superscripts depict the statistical difference ( $P < 0.05$ ) between means for the same yogurt batches at different time intervals

### 5.3.1.3 Texture profile analysis

The gel strength of the yogurt samples increased as a result of prebiotic addition (Table 5.3). Samples supplemented with sorbitol, mannitol, FOS and Synergy 1 showed an increase in yogurt firmness of up to 0.62, 0.63, 0.70, and 0.80 g, respectively during the storage period. This suggests that although supplementation of yogurt with different prebiotics could result in increased firmness, the gel strength increased considerably in the presence of Synergy 1. The effect of above supplementations on viscosity of yogurt samples was significantly increased ( $P < 0.05$ ) during the 21-day storage period. Walstra et al. (2006) stated that firmness and viscosity of set yogurt usually decrease as pH values increase, thus, considering the pH decline in this

study an increase in firmness and viscosity during the study period is justified. Improvement of textural properties of yogurt such as firmness and viscosity could improve sensory characteristics and overall palatability of the developed yogurt.

**Table 5.3** The effect of selected prebiotics on textural attributes of yogurt samples during storage

Prebiotic Storage Days		Firmness (N)	Consistency	Cohesiveness (Ns <sup>-1</sup> )	Viscosity (Pas <sup>-1</sup> )
<b>d1</b>	FOS	0.288±0.024 <sup>c</sup>	1.801±0.129 <sup>a</sup>	0.137±0.018 <sup>b</sup>	0.082±0.011 <sup>a</sup>
	Mannitol	0.296±0.037 <sup>bc</sup>	1.822±0.189 <sup>a</sup>	0.148±0.005 <sup>a</sup>	0.081±0.006 <sup>a</sup>
	Sorbitol	0.352±0.007 <sup>b</sup>	1.866±0.088 <sup>a</sup>	0.062±0.003 <sup>c</sup>	0.083±0.004 <sup>a</sup>
	Synergy1	0.371±0.008 <sup>a</sup>	1.888±0.040 <sup>b</sup>	0.143±0.001 <sup>a</sup>	0.081±0.004 <sup>a</sup>
	Control	0.266 ± 0.01 <sup>a</sup>	1.790± 0.05 <sup>a</sup>	0.070±0.001 <sup>b</sup>	0.076±0.003 <sup>a</sup>
<b>d7</b>	FOS	0.291±0.009 <sup>c</sup>	1.866±0.019 <sup>c</sup>	0.123±0.002 <sup>d</sup>	0.079±0.004 <sup>b</sup>
	Mannitol	0.330±0.040 <sup>b</sup>	2.160±0.037 <sup>b</sup>	0.138±0.013 <sup>c</sup>	0.081±0.004 <sup>b</sup>
	Sorbitol	0.385±0.030 <sup>ab</sup>	2.287±0.131 <sup>b</sup>	0.152±0.007 <sup>b</sup>	0.068±0.003 <sup>c</sup>
	Synergy1	0.394±0.004 <sup>a</sup>	2.943±0.047 <sup>a</sup>	0.159±0.010 <sup>a</sup>	0.095±0.004 <sup>a</sup>
	Control	0.286 ± 0.01 <sup>a</sup>	1.88 ± 0.05 <sup>a</sup>	0.098±0.001 <sup>b</sup>	0.086±0.003 <sup>a</sup>
<b>d14</b>	FOS	0.323±0.018 <sup>b</sup>	2.238±0.029 <sup>b</sup>	0.135±0.013 <sup>bc</sup>	0.080±0.002 <sup>b</sup>
	Mannitol	0.357±0.018 <sup>ab</sup>	2.041±0.001 <sup>c</sup>	0.130±0.001 <sup>c</sup>	0.085±0.004 <sup>b</sup>
	Sorbitol	0.321±0.030 <sup>b</sup>	2.391±0.177 <sup>b</sup>	0.145±0.002 <sup>a</sup>	0.093±0.000 <sup>a</sup>
	Synergy1	0.408±0.028 <sup>a</sup>	2.978±0.095 <sup>a</sup>	0.137±0.004 <sup>b</sup>	0.085±0.001 <sup>b</sup>
	Control	0.296 ± 0.01 <sup>a</sup>	1.901± 0.05 <sup>a</sup>	0.108±0.001 <sup>b</sup>	0.108±0.003 <sup>a</sup>
<b>d21</b>	FOS	0.351±0.047 <sup>c</sup>	2.052±0.100 <sup>b</sup>	0.152±0.001 <sup>b</sup>	0.089±0.002 <sup>b</sup>
	Mannitol	0.366±0.011 <sup>b</sup>	2.236±0.158 <sup>a</sup>	0.156±0.019 <sup>a</sup>	0.095±0.007 <sup>a</sup>
	Sorbitol	0.414±0.022 <sup>a</sup>	2.282±0.014 <sup>ab</sup>	0.140±0.008 <sup>d</sup>	0.089±0.001 <sup>b</sup>
	Synergy1	0.451±0.004 <sup>a</sup>	2.331±0.042 <sup>a</sup>	0.141±0.009 <sup>c</sup>	0.088±0.005 <sup>b</sup>
	Control	0.316 ± 0.01 <sup>a</sup>	2.001± 0.05 <sup>a</sup>	0.128±0.001 <sup>b</sup>	0.112±0.003 <sup>a</sup>

Results presented as a mean (n = 3) ± SD.

Different lower-case letter superscripts depict the statistical difference within a column, ( $P < 0.05$ ) between means for different yogurt batches.

### 5.3.2 Selection of appropriate concentration of Synergy 1 for yogurt production

The above results clearly indicate that among the four selected prebiotics, the supplementation of yogurt with 1.0 % (w/v) Synergy 1 resulted in a significant increase of probiotic count, optimum pH and an acceptable textural profile during incubation and storage

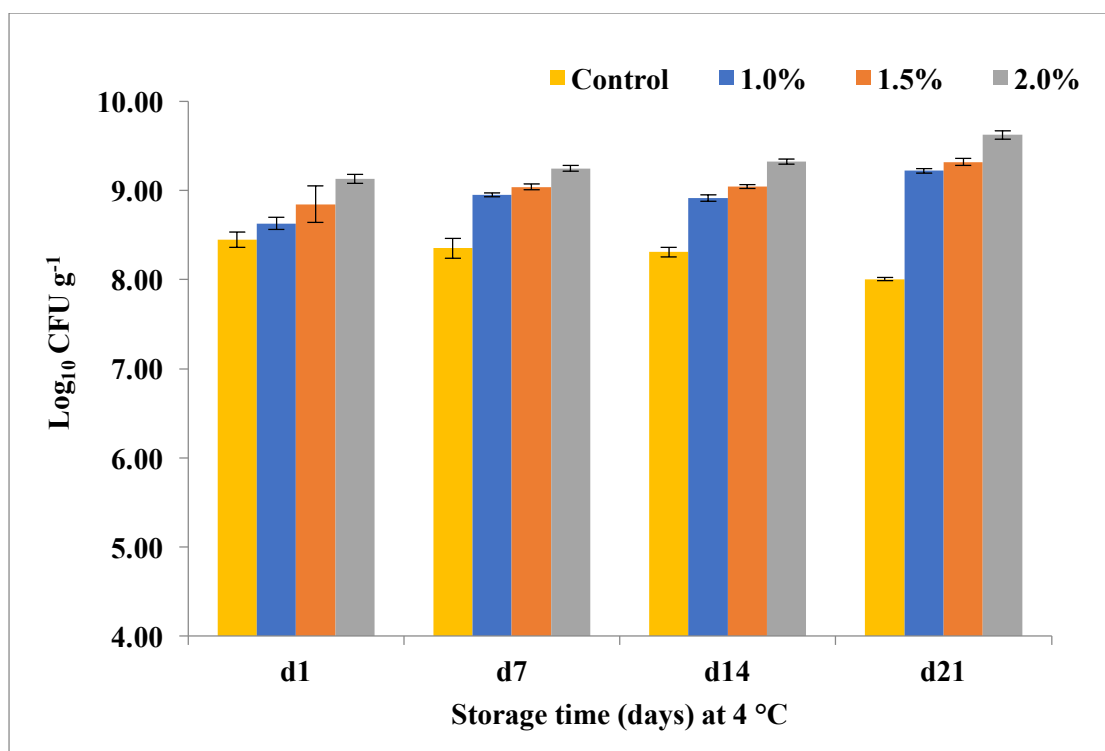
period and therefore it was selected for subsequent tests using different levels (0, 1, 1.5 and 2%). The concentration level with the optimal microbiological and physicochemical results was subsequently used in the development of synbiotic yogurt supplemented with a different concentration of PJC.

#### *5.3.2.1 Effect of Synergy 1 addition on LAB viable count*

The effects of different concentrations of Synergy 1 on LAB counts during 21-day storage at 4 °C is presented in Figure 5.2. Although there were minimal differences in the numbers of LAB in all yogurt samples, a slight increase was observed in yogurt supplemented with 1% and 1.5% of Synergy 1 throughout the storage period ( $P > 0.05$ ). The viable cell counts of LAB in the control sample declined from  $8.45 \pm 0.21 \text{ Log}_{10} \text{ CFU g}^{-1}$  on day 1 to  $8.01 \pm 0.19 \text{ Log}_{10} \text{ CFU g}^{-1}$  at the end of the storage period. In contrast, supplementation with 2% Synergy 1 resulted in a significant ( $P < 0.05$ ) increase in the total count of LAB from  $9.10 \pm 0.23$  to  $9.6 \pm 0.21 \text{ log}_{10} \text{ CFU g}^{-1}$  during refrigerated storage time.

The 2% (w/v) supplementation with Synergy 1 was the highest concentration used in this study in view of the fact that 2.0% (w/v) Synergy 1 could maintain the LAB viable counts in the synbiotic yogurt samples at  $9.6 \pm 0.21 \text{ log}_{10} \text{ CFU g}^{-1}$ , even at the higher level than the minimum level of  $10^6$ - $10^7 \text{ CFU g}^{-1}$  which is required to excrete the health effects, during the 21-day storage period. Therefore, it was not required to use higher concentrations of Synergy 1 in synbiotic yogurt development. Similar results reported by Akin et al. (2007) indicate that supplementation with inulin-type fructan at a rate of 2.0% (w/v), gave the best prebiotic effects. Ozer et al. (2005) also found that lactoluse powder, when added to yogurt up to 2.0%, could promote the counts of *L. acidophilus* LA-5 to a great extent.





**Figure 5.2** The effect of different concentrations of Synergy 1 on LAB growth in synbiotic yogurt during 21-day storage at 4°C.

#### 5.3.2.2 Effects of Synergy 1 addition on physicochemical parameters of yogurt

##### 5.3.2.2.1 Changes in pH values

The importance of pH measurement in the quality control of yogurt production has been mentioned in section 5.3.1.2.

The effect of Synergy 1 addition on pH values of yogurt samples during fermentation to reach the target pH was monitored. Fermentation was stopped near pH 4.7 in all samples, but the samples reached the previously mentioned pH in different times (data not shown). The study results showed that Synergy 1 supplementation did not have a significant impact on fermentation time of the yogurt samples and the pH values of yogurt samples supplemented with 1.0% and 1.5 % Synergy 1 were similar to the pH value of the control sample. Otherwise, the yogurt sample supplemented with 2.0% Synergy 1 had the shortest time of fermentation (250 min) compared to other tested samples (300 min). These findings are in agreement with

Stijepic et al. (2013) who reported no significant change in fermentation time of milk samples in the presence of different levels of inulin supplementation. The cow's milk sample with 1.5% inulin had the shortest time of fermentation (250 min) compared to all the tasted samples. This finding could lead to further research into the optimal percentage of inulin supplementation.

Kusuma et al (2009) have shown that the yogurt gel made with lower pre-acidification pH and longer fermentation time had lower storage modulus, higher whey separation and permeability, and a grainy texture. In addition, longer fermentation time resulted in a lower rate of bond formation, weaker gels and the presence of large pores in the gels and overall reduction in consumer acceptability (Kusuma et al., 2009). Therefore, the food industry generally favours short fermentation times during processing and the results of this study suggest that reducing the fermentation time improved gel properties (Kusuma et al., 2009; Peng et al., 2009).

Changes in pH during refrigerated storage of yogurt samples supplemented with different levels of Synergy 1 are shown in Table 5.2. The pH values declined slowly in all samples until the end of the first week but thereafter declined more significantly ( $P < 0.05$ ) in yogurt samples supplemented with 1.5 % (w/v) and 2.0% (w/v). At the end of the first week of refrigerated storage, the pH values of all yogurt samples were relatively consistent and not significantly different from each other. At the end of the second week (d8-d14), the control samples and yogurt samples with 1.0% (w/v) Synergy 1 did not show any significant decline in pH, while samples with 1.5% (w/v) and 2.0 % (w/v) Synergy 1 showed a significant ( $P < 0.05$ ) decline in pH by day 14 of storage. On the twenty-first day, there was no significant drop in pH between control samples and those supplemented with 1.0 % (w/v) Synergy1; however, yogurts with 2.0% (w/v) showed the lowest mean pH values (Table 5.4).

**Table 5.4** The effect of different levels of Synergy 1 on pH of the yogurt samples during storage

Storage (days)	Level of Synergy 1 Supplementation			
	0% (Control)	1%	1.5%	2%
<b>d1</b>	4.65 ± 0.021 <sup>aA</sup>	4.69 ± 0.012 <sup>aA</sup>	4.66 ± 0.032 <sup>aA</sup>	4.67 ± 0.042 <sup>aA</sup>
<b>d7</b>	4.61 ± 0.012 <sup>aA</sup>	4.60 ± 0.010 <sup>aA</sup>	4.53 ± 0.010 <sup>aA</sup>	4.59 ± 0.015 <sup>aA</sup>
<b>d14</b>	4.58 ± 0.006 <sup>aB</sup>	4.52 ± 0.006 <sup>aB</sup>	4.47 ± 0.006 <sup>aB</sup>	4.48 ± 0.072 <sup>bB</sup>
<b>d21</b>	4.52 ± 0.010 <sup>aC</sup>	4.50 ± 0.015 <sup>aB</sup>	4.34 ± 0.015 <sup>aB</sup>	4.20 ± 0.040 <sup>bC</sup>

Results presented as a mean (n = 3) ± SD.

Different lower -case letter superscripts depict the statistical difference within a row, ( $P < 0.05$ ) between means for different yogurt batches.

Different capital letter superscripts depict the statistical difference

( $P < 0.05$ ) between means for the same yogurt batches at different time intervals

#### 5.3.2.2.2 Texture of yogurt

The texture profile of yogurt samples supplemented with different concentrations of Synergy 1 during 21-day storage at 4°C is presented in Table 5.5. The results showed that the addition of Synergy 1 to the developed yogurt altered its textural properties. Synergy 1-containing yogurts normally showed a lower firmness than the control (without inulin). The firmness of yogurt is directly dependent on its total solids and specifically, protein content and the type of proteins. A higher protein content would cause a higher degree of cross linking of the gel network, resulting in a much denser and more rigid gel structure (Paseephol et al., 2008). In the current study, the yogurt samples were prepared by reconstituting the LHSMP in distilled water (Table 5.1, section 5.2.2.3), where control yogurt (0% Synergy 1) was standardised to 16% total solids using LHSMP, while the yogurt samples supplemented with 1%, 1.5% and 2% Synergy 1 were prepared by 15%, 14.5% and 14% LHSMP, respectively, which resulted in lower net protein content compared to the control. Besides this, the molecules of Synergy 1 are dispersed amongst the casein micelles, thus interfering with protein matrix formation

(Kusuma et al., 2009). These facts are believed to be responsible for a softer yogurt gel formation. The findings of an earlier study by Paseephol et al. (2008) which found a significant impact of inulin supplementation on the firmness of fat-free yogurt seems to support our study results.

Gel strength and viscosity are important quality indicators related to consistency and mouth feel of fermented dairy products (Lewis, 1996) and stability of viscosity during the storage is an important qualitative characteristic of yogurt. According to Rawson and Marschall (1997), *Streptococcus thermophilus* have an important role in the production of exocellular texturing agents called exopolysaccharides (EPS) that might interact with the protein content of milk and increase the viscosity and rheological quality of products. Viscosity of the yogurt samples during the storage period is also presented in Table 5.3. No differences were noticed among the viscosities up to fourteen days of storage. However, after the fourteenth day, the yogurt samples with added Synergy 1 showed less stability and among them, the yogurt samples with 1.5% and 2.0% Synergy 1 were significantly different from the control ( $P < 0.05$ ). All the statistically different samples showed decreased of viscosity after Synergy 1 addition, which is in agreement with the findings of several researchers (Kusuma et al., 2009; Paseephol & Sherkat, 2009).

All of the results reported herein indicate that supplementation of yogurt with 2.0% (w/v) Synergy 1 resulted in the optimal microbiological numbers and physicochemical properties over the storage period and therefore,

All the results reported herein indicate that supplementation of yogurt with 2.0% (w/v) Synergy 1 resulted in the optimal microbiological numbers and physicochemical properties over the storage period and therefore, there was not any need to increase the prebiotic supplementation level. In addition, the synbiotic yogurt supplemented with 2.0% (w/v) Synergy 1 can be used as the control sample where different levels of PJC (10, 12.5, 15 and

20%) were added to the sybiotic yogurt. The results of total phenolic compounds, antioxidant and proteolytic activity of the synbiotic yogurt supplemented with 2.0% (w/v) are in Appendix 5.5.

**Table 5.5** Texture profile of yogurt supplemented with different concentration of Synergy 1 during 21-day storage at 4°C

Storage days	Synergy 1	Firmness (N)	Consistency	Cohesiveness (Ns <sup>-1</sup> )	Viscosity (Pas <sup>-1</sup> )
<b>d1</b>	0% Control	0.362±0.21 <sup>a</sup>	2.110±0.17 <sup>b</sup>	0.155±0.01 <sup>b</sup>	0.066±0.06 <sup>a</sup>
	1.0%	0.397±0.27 <sup>a</sup>	2.094±0.17 <sup>b</sup>	0.143±0.01 <sup>b</sup>	0.058±0.04 <sup>a</sup>
	1.5%	0.480±0.14 <sup>a</sup>	2.036±0.81 <sup>b</sup>	0.154±0.01 <sup>b</sup>	0.064±0.07 <sup>a</sup>
	2.0%	0.523 ±0.17 <sup>a</sup>	2.902±0.18 <sup>a</sup>	0.195±0.03 <sup>a</sup>	0.069±0.03 <sup>a</sup>
<b>d7</b>	0% Control	0.351±0.30 <sup>a</sup>	2.151±0.32 <sup>a</sup>	0.158±0.03 <sup>a</sup>	0.067±0.02 <sup>a</sup>
	1.0%	0.371±0.48 <sup>a</sup>	2.210±0.28 <sup>a</sup>	0.150±0.03 <sup>a</sup>	0.056±0.02 <sup>a</sup>
	1.5%	0.343±0.34 <sup>a</sup>	1.976±0.32 <sup>a</sup>	0.143±0.03 <sup>a</sup>	0.062±0.02 <sup>a</sup>
	2.0%	0.362±0.48 <sup>a</sup>	2.798±0.28 <sup>a</sup>	0.172±0.03 <sup>a</sup>	0.067±0.01 <sup>a</sup>
<b>d14</b>	0% Control	0.360±0.08 <sup>b</sup>	2.186±0.18 <sup>b</sup>	0.159±0.02 <sup>a</sup>	0.066±0.01 <sup>a</sup>
	1.0%	0.383±0.11 <sup>b</sup>	2.517±0.05 <sup>b</sup>	0.167±0.05 <sup>a</sup>	0.052±0.04 <sup>a</sup>
	1.5%	0.376±0.18 <sup>b</sup>	2.224±0.08 <sup>b</sup>	0.160±0.01 <sup>a</sup>	0.055±0.04 <sup>b</sup>
	2.0%	0.496±0.15 <sup>a</sup>	2.248±0.90 <sup>a</sup>	0.160±0.04 <sup>a</sup>	0.060±0.01 <sup>b</sup>
<b>d21</b>	0% Control	0.345±0.34 <sup>b</sup>	2.212±0.15 <sup>a</sup>	0.164±0.05 <sup>a</sup>	0.065±0.02 <sup>a</sup>
	1.0%	0.369±0.15 <sup>b</sup>	2.417±0.11 <sup>a</sup>	0.176±0.08 <sup>a</sup>	0.049±0.05 <sup>a</sup>
	1.5%	0.332±0.13 <sup>c</sup>	2.197±0.07 <sup>a</sup>	0.150±0.08 <sup>a</sup>	0.069±0.02 <sup>c</sup>
	2.0%	0.346±0.26 <sup>c</sup>	2.430±0.90 <sup>a</sup>	0.161±0.05 <sup>a</sup>	0.055±0.01 <sup>c</sup>

Data represents the mean ± SD of triplicate analyses from each trial. The different letter superscripts depict the statistical difference within each column,  $p < 0.05$  between means for the same yogurt batches at different time intervals.

Control: without synergy

### 5.3.3 Effect of PJC addition on LAB viable count

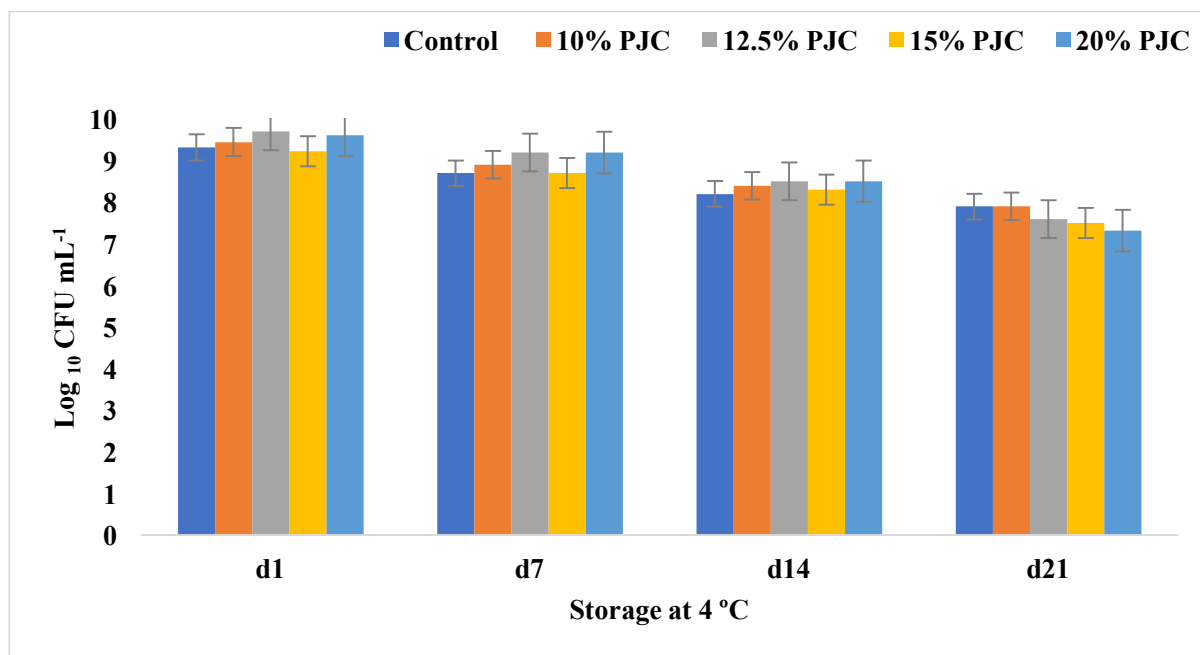
A synbiotic yogurt supplemented with PJC was developed by adding different levels of PJC (10, 12.5, 15 and 20%) to sterilized milk before or after heat treatment at 90 °C for 10 min. Supplementation before heat treatment was limited to 10%, since above this level the milk curdled, whereas after heat treatment, up to 20% PJC could be added without any adverse effect. It appeared that heat treatment of milk increased its stability and buffering capacity. Effects of PJC addition on the viable counts of *L. rhamnosus* 1520 and *L. acidophilus* 2404 in the control and PJC-supplemented yogurts during the storage period is presented in Figure 5.3.

At the beginning of storage (day 1), the initial population of LAB were over  $9 \text{ Log}_{10} \pm 0.06 \text{ CFU mL}^{-1}$  in all yogurt samples. After seven days, the LAB numbers in the control sample decreased by 0.7  $\text{Log}_{10}$  cycle and did not significantly changed during the rest of the storage period.

Supplementation with PJC slightly decreased the number of LAB in the first week of storage. During the last week of storage, LAB viable counts decreased significantly ( $P < 0.05$ ) in all yogurt samples which contained PJC ( $P < 0.05$ ), while in yogurt containing 20.0 % PJC the drop was more noticeable.

Considering that these yogurt samples were provided to the study subjects on one to two days post product manufacturing, the LAB population at the point of consumption ( $7.2 \text{ Log}_{10} \pm 0.26 \text{ CFU mL}^{-1}$ ) was still within the acceptable range.

This observation was consistent with the findings of Arjmand (2011) who reported that supplementation of yogurt samples with 6% PJC had a minimal effect on the population of La-5 up to week 3 (ca. 0.1  $\text{Log}_{10}$  cycle). However, in the last week, the population of La-5 dropped significantly and the final count showed the ca. 0.2  $\text{Log}_{10}$  cycle less than the control sample.



**Figure 5.3** Effects of PJC addition on LAB viable count (CFU  $\text{Log}_{10} \text{mL}^{-1}$ ) in synbiotic yogurt during storage at 4°C.

### 5.3.4 Effect of PJC addition on physicochemical parameters of synbiotic yogurt

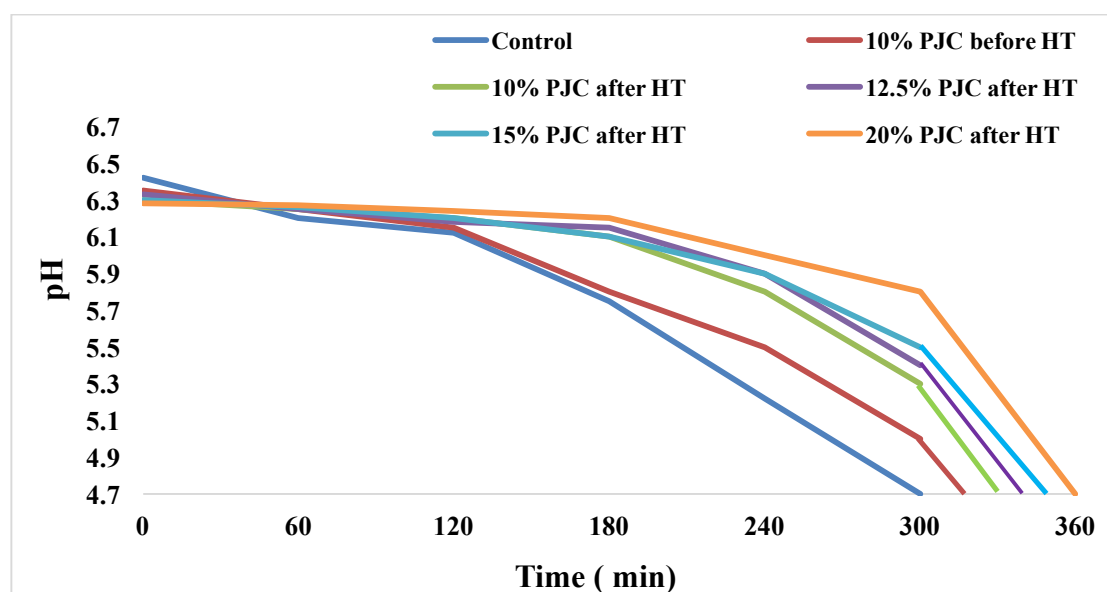
#### 5.3.4.1 pH trend

Increasing the level of PJC supplementation to 20% depressed the milk pH to 6.28 (Figure 5.4). After inoculation with probiotic cultures, the samples were incubated at 43 °C to reach pH 4.7, at which point the time was recorded and yogurt samples were transferred to refrigerated storage at 4 °C.

The activity of the starter culture is usually estimated from the time it takes to reach the target pH. Comparing the time taken to reach pH 4.7 in the control yogurt and the supplemented samples, it became evident that PJC supplementation has a negative influence on the activity of starter cultures that delays the yogurt setting time. The control yogurt reached the target pH after 300 minutes while the supplemented samples with 10, 12.5, 15, and 20% showed 40, 45, 50, and 60 minutes' delay reaching the target pH, respectively. However, the apparent short

setting time of samples containing 15 and 20% PJC is mainly due to the low starting pH of these samples, caused by PJC addition, therefore, they cannot be considered as positive results.

These results further confirmed that PJC supplementation could reduce the starter culture activity during the incubation time. Therefore, a more thorough microbiological study was undertaken to identify the PJC impact on the activity and survival of these bacteria (section 5.3).



**Figure 5.4** pH changes in yogurts supplemented with PJC before and after heat treatment (The data represent the means  $\pm$  SD of triplicate analyses from each trial)

#### 5.3.4.2 Texture of yogurt

Changes in textural attributes of yogurt as a result of PJC addition during storage at 4°C for twenty-one days is presented in Table 5.6. The result showed that the addition of PJC had a dilution effect on yogurt composition and altered its textural properties. On day 1 of storage, PJC containing yogurt showed a significantly lower firmness than the control (without PJC) ( $P < 0.05$ ). The firmness of yogurt is directly dependent on its total solids and specifically, protein content and the type of proteins. Higher protein content would cause a higher degree of cross-linking of the gel network, resulting in a much denser and more rigid gel structure (Paseephol



et al., 2008). In the current study, the amount of protein in milk was kept constant (Table 5.1, section 5.2.2.3); however, in PJC-supplemented yogurts, extra acid in PJC might have hydrolysed some of the casein, which resulted in lower net protein content compared to the control. The findings of earlier studies stating the significant impact of fruit or vegetable juice concentrate supplementation on the firmness of yogurt seems to support our study results (Arjmand, 2011; Kiros et al., 2016).

Gel strength and viscosity are important quality indicators related to consistency and mouth feel of fermented dairy products (Lewis, 1996) and stability of viscosity during the storage is an important qualitative characteristic of yogurt. Viscosity of yogurt samples during the period of storage is also presented in Table 5.5. Significant differences were noticed among the viscosities of yogurt samples during the storage period and samples supplemented with 20% PJC showed decreasing of viscosity during storage ( $P < 0.05$ ). All of the statistical differenced samples, which is in agreement with the findings by Arjmand (2011) who also confirmed that supplementation of probiotic yogurts with 6% PJC resulted in inferior textural properties compared to control samples.

**Table 5.6** Changes in textural attributes of yogurt as a result of PJC addition during 21-day storage at 4°C

Storage	PJC Levels	Firmness (N)	Consistency	Cohesiveness (Ns <sup>-1</sup> )	Viscosity(Pas <sup>-1</sup> )
<b>d1</b>	0% (control)	0.346±0.02 <sup>a</sup>	2.902±0.18 <sup>a</sup>	0.195±0.03 <sup>a</sup>	0.069±0.03 <sup>a</sup>
	20%	0.092±0.05 <sup>b</sup>	0.617±0.02 <sup>a</sup>	0.065±0.02 <sup>b</sup>	0.030±0.01 <sup>b</sup>
<b>d7</b>	0% (control)	0.322±0.04 <sup>a</sup>	2.798±0.28 <sup>a</sup>	0.172±0.03 <sup>a</sup>	0.065±0.01 <sup>a</sup>
	20%	0.088±0.02 <sup>b</sup>	0.594±0.01 <sup>a</sup>	0.068±0.02 <sup>b</sup>	0.027±0.02 <sup>b</sup>
<b>d14</b>	0% (control)	0.296±0.15 <sup>a</sup>	2.448±0.90 <sup>a</sup>	0.160±0.04 <sup>a</sup>	0.065±0.15 <sup>a</sup>
	20%	0.073±0.06 <sup>c</sup>	0.623±0.03 <sup>a</sup>	0.071±0.07 <sup>b</sup>	0.025±0.04 <sup>b</sup>
<b>d21</b>	0% (control)	0.123 ±0.16 <sup>a</sup>	2.230±0.90 <sup>a</sup>	0.161±0.05 <sup>a</sup>	0.072±0.17 <sup>a</sup>
	20%	0.065±0.06 <sup>d</sup>	0.629±0.03 <sup>a</sup>	0.072±0.03 <sup>b</sup>	0.023±0.03 <sup>c</sup>

Data represents the mean ± SD of triplicate analyses from each trial. The different letter superscripts in each column show a statistical significant difference  $P < 0.05$ .

Control: without PJC

### 5.3.4.3 Determination of antioxidant activity

The scavenging effects of yogurt samples supplemented with different levels of PJC on DPPH radical are shown in Table 5.7. In DPPH radical scavenging assay, the percentage of scavenging increased with the increase in PJC supplementation. However, the AA in PJC-supplemented yogurts decreased continuously over a storage period from one to twenty-one days, which may be attributed to denaturation during the storage period as well as the storage temperature. Lawin and Kongbangkerd (2010) reported a decrease in antioxidant activity of yogurts fortified with roselle syrup after six days of storage due to loss of anthocyanin activity.

**Table 5.7** Scavenging effects of yogurt samples supplemented with different levels of PJC on DPPH radical

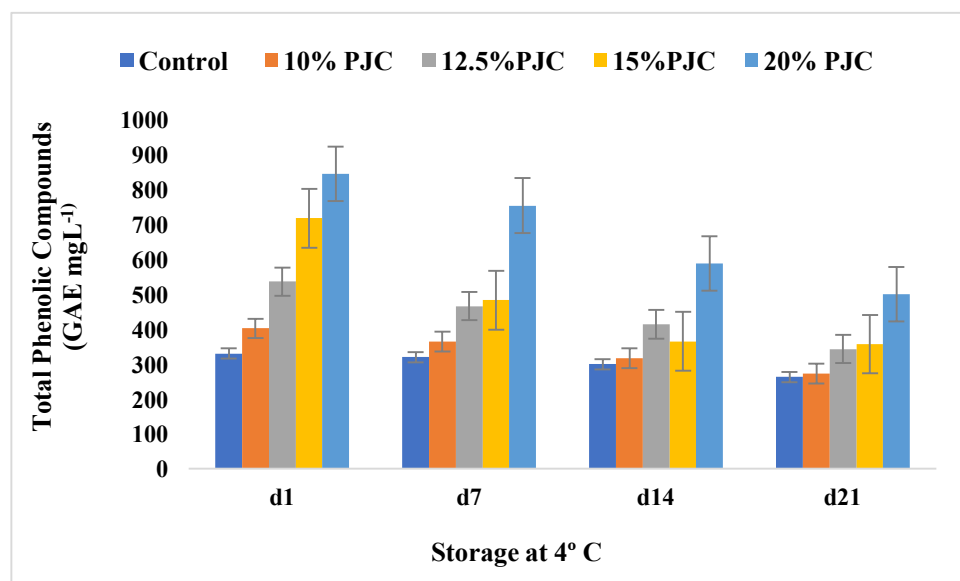
%PJC addition Storage (days)	DPPH Inhibition (%)				
	0% (Control)	10.0 %	12.50 %	15.0 %	20.0 %
<b>d1</b>	61.5±0.30 <sup>a</sup>	77.0±0.11 <sup>a</sup>	82.0± 0.06 <sup>a</sup>	85.0± 0.06 <sup>a</sup>	93.0±0.30 <sup>b</sup>
<b>d7</b>	62.3±0.02 <sup>a</sup>	72.0±0.19 <sup>a</sup>	79.8± 00.06 <sup>a</sup>	81.8± 00.06 <sup>a</sup>	90.8±0.13 <sup>b</sup>
<b>d14</b>	63.0±0.06 <sup>a</sup>	69.4±0.06 <sup>a</sup>	77.1± 0.03 <sup>a</sup>	78.1± 0.03 <sup>a</sup>	86.1±0.07 <sup>b</sup>
<b>d21</b>	63.2±0.01 <sup>a</sup>	67.0±0.03 <sup>a</sup>	74.8±0.01 <sup>a</sup>	76.8±0.01 <sup>a</sup>	82.0±0.01 <sup>b</sup>

Data represents the mean ± SD of triplicate analyses from each trial. The small letter superscripts depict the statistical difference within each column,  $p < 0.05$  between means for the same yogurt batches at different time intervals.

### 5.3.4.4 Total phenolic compounds (TPCs)

In synbiotic yogurt samples, the amount of TPCs initially improved significantly ( $P < 0.05$ ) with an increase in concentration of PJC ( $402 \pm 0.08$ ,  $536 \pm 0.05$ ,  $718 \pm 0.06$ , and  $845 \pm 0.01$  GAE mg L<sup>-1</sup> in yogurt samples containing 10.0, 12.50, 15.0 and 20.0% (v/v) PJC, respectively); however, these amounts declined to  $272 \pm 0.03$ ,  $343 \pm 0.01$ ,  $357 \pm 0.04$ , and  $500 \pm 0.01$  GAE 100 mg L<sup>-1</sup>, respectively at the end of the storage period (Figure 5.5). The observed values of TPCs in the present study were similar to earlier studies Arjmand (2011) and El-Said et al.

(2014). Both studies reported a significant increase in the amount of TPCs with an increased PJC supplementation in synbiotic yogurts on day 1 of the storage. However, the TPC declined at the end of the storage period. This reduction could be due to the degradation of phenolic compounds during the storage period (Yildiz & Eydurán, 2009) or increasing the milk protein-polyphenol interaction (Yuksel et al., 2010).

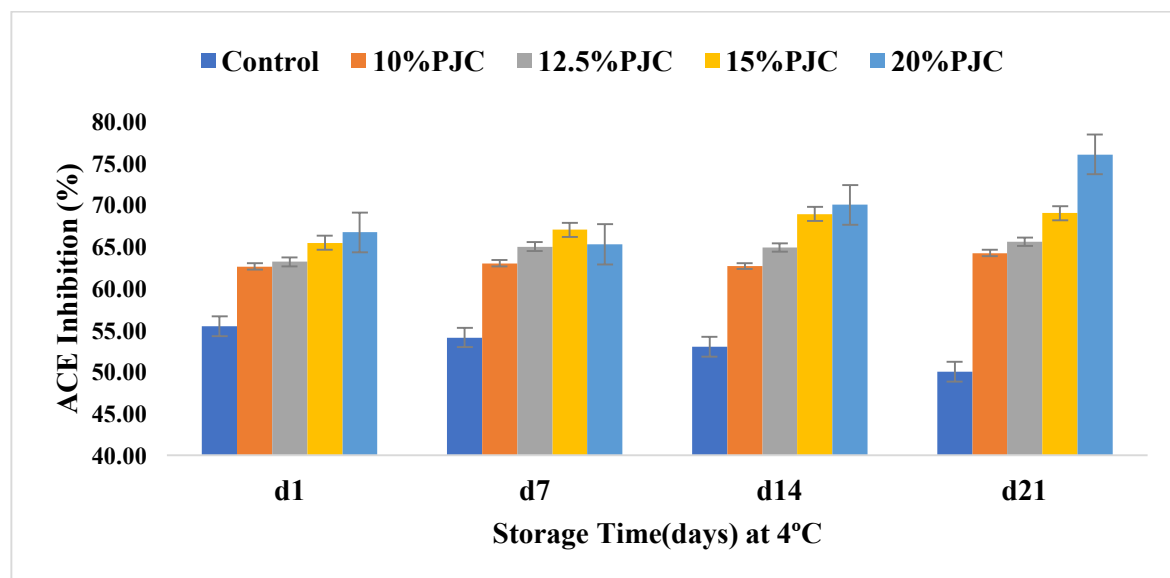


**Figure 5.5** Degradation of phenolic compounds in synbiotic yogurt supplemented with different concentrations of PJC during 21-day storage at 4°C.

#### 5.3.4.5 Antihypertensive properties

The ACE-inhibitory activity of the synbiotic yogurt supplemented with different PJC concentrations are illustrated in Figure 5.6. The ACE-inhibition activity significantly increased ( $P < 0.05$ ) during the 21-day storage period in all synbiotic yogurts which may be due to the proteolytic activity of LAB. This proteolytic activity usually employs an extracellular serine protease to degrade casein and produce oligopeptides, which may inhibit the ACE activity and provide an antihypertensive effect (Beganovic et al., 2013). This implies that our synbiotic yogurt had a potential antihypertensive effect. Furthermore, the results showed that in comparison with all other synbiotic yogurt samples, those supplemented with 20% PJC, due to

the combined effects of small peptides produced by the probiotics and the presence of high levels of TPCs, had a significantly increased ACE-inhibition activity by 75% throughout the storage period ( $P < 0.05$ ).



**Figure 5.6** ACE-inhibitory properties of synbiotic yogurt supplemented with different levels of PJC during storage at 4°C for 21 days

#### 5.3.4.6 Colour measurement

As expected, all colour parameters were significantly ( $P < 0.05$ ) different between the control (C) and supplemented samples. The addition of 20% PJC with low  $L^*$  ( $18.41 \pm 0.22$ ),  $b^*$  ( $1.21 \pm 0.01$ ),  $c$  ( $3.81 \pm 0.06$ ) and  $H^\circ$  ( $17.15 \pm 0.32$ ) values, and high  $a^*$  ( $14.45 \pm 0.08$ ) and colour index ( $8.03 \pm 0.08$ ) values changed the milky white colour of the sample to pink. This was demonstrated by a significant ( $P < 0.05$ ) increase in  $a^*$  value from - 4.17 to +6.85. At the same time, the luminosity ( $L^*$ ) and colour intensity ( $C$ ) of samples decreased significantly ( $P < 0.05$ ). According to  $H^\circ$  formula [ $H^\circ = \tan^{-1} (b^*/a^*)$ ], any increase in the redness of a sample ( $a^*+$ ) or drop in its yellowness ( $b^*+$ ) results in low  $H^\circ$  value. The  $H^\circ$  value of SynP (36.83) was significantly ( $P < 0.05$ ) lower than the control sample (112.24), while its colour index value was significantly ( $P < 0.05$ ) higher (1.63 vs. 0.57). These results correlated with visual

observation of the samples, indicating that the samples which contained PJC had a higher colour index and an attractive, bright pink appearance.

#### 5.3.4.7 *Sensory evaluation of synbiotic yogurt supplemented with 20% PJC*

As described in section 5.3.4.1, a synbiotic yogurt supplemented with PJC was developed by incorporating different levels of PJC (10, 12.5, 15 and 20%), added to standardised milk before or after heat treatment at 90 °C for 10 min. To compensate for the dilution effect of PJC addition, the protein content of these samples was kept constant by adjusting the amount of LHSMP (14%) added to their formulations (Section 5.2.2.2; Table 5.1).

Supplementation with 20% PJC revealed the optimal microbiological and physicochemical results over the storage period, therefore, this sample was suitable for sensory evaluation by an untrained panel (n=25) for attributes such as aroma, colour, appearance, gel thickness and firmness, flavour and overall acceptability. Although, the panel size was small, however the panelists were provided by 2 × 1h sensory training sessions prior the study. The average sensory scores of different yogurt samples are presented in Table 5.8.

The addition of 20% PJC to the formulation of synbiotic yogurt (SynP) compared to non-PJC added synbiotic (Syn) and control (C) samples had a significant impact on all tested sensory attributes, except the gel thickness and gel firmness ( $P < 0.05$ ) (Fig.5.11). One of the most obvious effects of PJC supplementation was the development of a grainy texture and softer gel in PJC- supplemented yogurts as a result of interaction between casein and the acids in PJC during incubation. As expected, due to a higher PJC level in sample SynP, panelists were able to perceive a stronger fruity aroma and flavour with a deeper pink colour (Fig. 5.7). Overall acceptance scores revealed that sample SynP was significantly ( $P < 0.05$ ) more desirable to the panellists than sample Syn.

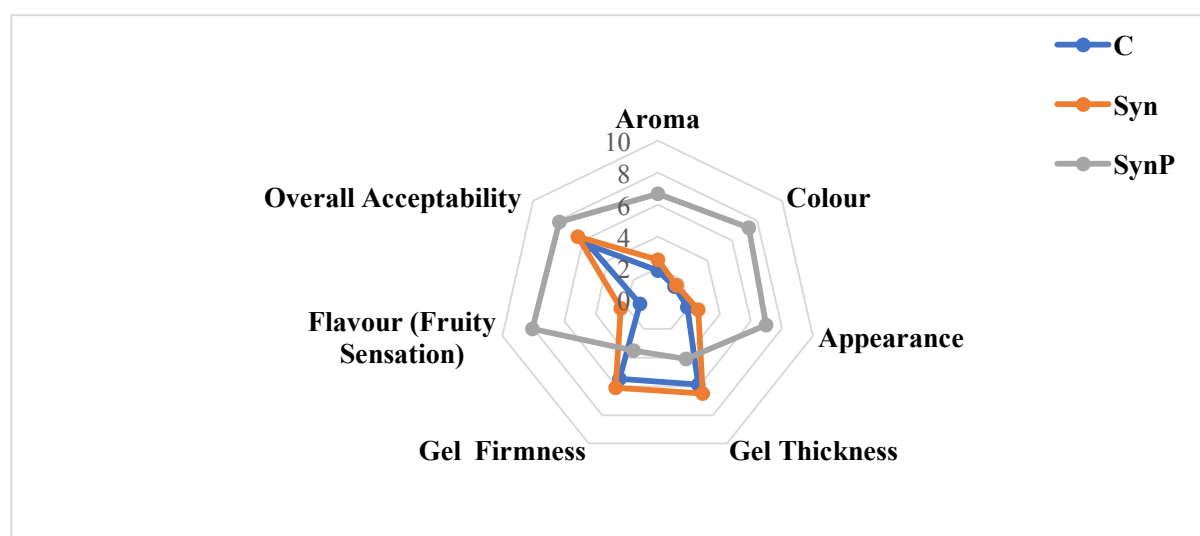
Results obtained indicated that although sample SynP presented a slightly grainy and soft gel, its deeper pink colour and stronger fruity aroma and flavour resulted in higher acceptability among the panelists in comparison to other samples (Figure 5.7).

**Table 5.8** Sensory attributes of yogurt samples developed in this study

Samples	Aroma	Colour	Appearance	Gel thickness	Gel firmness	Fruity flavour	Overall acceptability
Control	1.85± 1.3 <sup>a</sup>	1.37±1.2 <sup>a</sup>	1.9±1.1 <sup>a</sup>	5.87±1.2 <sup>b</sup>	5.50 ±1.2 <sup>b</sup>	1.15±0.5 <sup>b</sup>	5.52±1.5 <sup>c</sup>
Syn <sup>1</sup>	2.55±0.94 <sup>b</sup>	1.50±1.7 <sup>b</sup>	2.6±1.5 <sup>b</sup>	6.47±1.0 <sup>a</sup>	6.10 ±1.3 <sup>a</sup>	1.37±0.6 <sup>b</sup>	6.40±1.9 <sup>c</sup>
SynP <sup>2</sup>	6.67±1.2 <sup>c</sup>	7.30±1.3 <sup>b</sup>	7.0±1.1 <sup>c</sup>	4.10±1.3 <sup>c</sup>	3.52 ± 1.2 <sup>c</sup>	8.07± 1.8 <sup>a</sup>	7.90±1.5 <sup>c</sup>

Data represents the mean ± SD of triplicate analyses from each trial. The different letter superscripts in each column show a statistical significant difference  $P < 0.05$ ,  $n = 25$ .

<sup>1</sup> synbiotic yogurt <sup>2</sup> synbiotic yogurt with 20% PJC supplementation



**Figure 5.7** Polar presentation of sensory data (n=25)

## 5.4 CONCLUSION

This study involved the development of a synbiotic yogurt by incorporating PJC as a supplement to be used in human feeding trials in order to quantify the hypocholesterolemic and antihypertensive effects of probiotics on hypercholesterolemic men and women. In addition,

the effects of different percentages of PJC supplementation in different stages of the yogurt making procedure were analysed. The results indicated up to 20% PJC could be supplemented post-heat treatment, and no apparent antagonism was observed between the cultures' activity and the PJC phytochemicals during the incubation time. While strong astringency of PJC could limit its application for direct consumption, pH-adjusted PJC may find uses in nutraceuticals as an AA supplement.

Further analyses on the synbiotic yogurt supplemented with PJC indicated that, although it showed inferior textural properties compared to the control sample, it had a higher colour index and contained over 63%, 94% and 75% more TPC, AA and ACE inhibition activity, respectively. In addition, (n=25) untrained sensory panellists was a small sample size.

The sensory evaluation of the PJC-enriched synbiotic yogurt revealed that by increasing the PJC level the overall acceptability among the panellists increased significantly and therefore, this product could offer a pleasant and effective route to increasing the antioxidant intake in our daily diet. Despite the slight adverse effect of PJC supplementation at 20% level on probiotic numbers, the probiotic population at the end of 21-day storage period at 4 °C was still within the acceptable range ( $> 10^6$  CFU mL<sup>-1</sup>) to deliver health-promoting properties.

Considering the strong astringency of PJC, despite its high amount of TPC and AA, further investigations are required to use pomegranate and its derivatives as AA supplements in other functional foods. In addition, in order to overcome the textural problems of the developed synbiotic yogurt, more investigation is needed into yogurt formulation and manufacturing.

In conclusion, the selected probiotics *L. acidophilus* CSCC 2404 and *L. rhamnosus* ASCC 1520 initially proved to have the ability to tolerate acid and bile conditions (refer to sections 3.3.1 and 3.3.2). Moreover, they were optimised with 2.0% (w/v) Synergy 1 and 20% (v/v) PJC for maximum cholesterol removal and hypotensive properties in a human feeding trial to substantiate the health benefits of the synbiotic product developed in this project.

**6. THE EFFECT OF SYNBIOTIC YOGURT CONTAINING  
POMEGRANATE POLYPHENOLS ON LIPID PROFILE IN  
INDIVIDUALS WITH HYPERLIPIDAEMIA: A  
RANDOMISED CONTROLLED CLINICAL TRIAL**



## 6.1 INTRODUCTION

Many epidemiologists have argued that economic development pushes populations through a nutrition transition, shifting food preferences from traditional diets characterized by low saturated fat, to less healthy diets (Popkin et al., 2012; Shridhar et al., 2015). Unhealthy diets cause obesity with increased risk of high cholesterol and fatty streak development, leading to greater risk of CVD (Tuso et al., 2013). A large majority of epidemiological studies have identified the elevated serum total cholesterol (TC), high level of low density lipoprotein (LDL), and elevated plasma triglycerides (TG) as risk factors for atherosclerosis and CVD (Chen et al., 2011). Indeed, high fat diet is regarded as an important factor in the development of atherosclerosis, hypercholesterolemia (HCE), hypertension (HT), and ischemic heart disease (IHD).

Nowadays, consumers have become more aware of the relationship between good health and food intake, particularly from naturally derived foods such as fruits, vegetables and low-fat probiotic dairy products. The concept of "functional food" involves and requires the use of bioactive ingredients or the presence of natural healthy bioactive molecules in foods (Gilliland, 1979; Gibson & Roberfroid, 1995; Roberfroid, 2000) .

Yogurt is the most popular dairy product as most consumers consider it a 'healthy product' having desirable health effects (Gilliland, 1979; Ziemer & Gibson, 1998; Roberfroid, 2000) (Gibson & Fuller, 2000; Lourens-Hattingh & Viljoen, 2001). Manufacturers of yogurt and yogurt-like products have been marketing and modifying successfully to meet consumers' demands (Gonzalez et al. 2011). The increasing yogurt consumption trends in many countries have been attributed to the increased variety of fruit-flavoured yogurt in markets (Gonzalez et al., 2011).

Fruit-flavoured yogurts are manufactured by adding fruit concentrates or flavoured syrups to the cultured milk before or after the incubation process. Pomegranate (*Punica granatum L.*)

is an important fruit of tropical and subtropical regions, which originated in the Middle East and India and it is one of the oldest known fruit. It is widely reported that pomegranate exhibits antiviral, antioxidant, anti-cancer, and anti-proliferative properties on tumour cells (Lansky et al., 1998). Pomegranate is consumed fresh and in processed form as juice (PJ), wines, flavours, and extracts. Fresh PJ has the highest antioxidant activities compared to other fruit juices, red wine and green tea. Compared to the experimental juices obtained only from the arils, PJ is considered as one of the richest sources of polymeric polyphenols with maximum antioxidant capacity due to the presence of punicalagin ( $1.56 \text{ gL}^{-1}$ ); ellagitannins (ETs) ( $2.4 \text{ gL}^{-1}$ ); free anthocyanins ( $0.39 \text{ gL}^{-1}$ ), ellagic acid ( $0.12 \text{ gL}^{-1}$ ), hydrolysable tannin ( $0.42 \text{ gL}^{-1}$ ), and total phenolic compounds ( $2.5 \text{ gL}^{-1}$ ) (Sakr & Abou Dawood, 2015). However, approximately 50% of the total fruit weight, which contains a significant portion of polyphenol compounds corresponds in the peels, is usually discarded as waste. Ellagic acid and hydrolysable ellagitannins are both implicated in protection against atherogenesis, along with their potent antioxidant capacity. Punicalagin is the major ellagitannin responsible for the high antioxidant activity of the juice. As a major source of polyphenolics, PJ was shown to be a very potent antioxidant against LDL oxidation as it was shown to inhibit the atherosclerosis development in mice and humans (Aviram, 2002; Rosenblat & Aviram, 2006; Al-Zoreky, 2009; Arjmand, 2011).

Although some human and animal studies have suggested moderate hypocholesterolemic effects of some fermented dairy products (St-Onge et al., 2000; Kieling et al., 2002; Ohlsson, 2010), there seems to be a lack of clinical evidence on the effect of synbiotic yogurt on cholesterol reduction and blood pressure (BP). Therefore, the aim of this study was to provide credible evidence on the effects of the PJ-supplemented synbiotic yogurt developed in our laboratories (Chapter 5, section 5.2.2.), on serum lipid profile and BP in mildly to moderately hypercholesterolemic and hypertensive subjects.

## **6.2 MATERIALS AND METHODS**

### **6.2.1 Subject selection and study method**

A total of forty-eight male and female volunteers were recruited from the Northend Medical Centre (54 Childs Road, Epping 3076, Australia). All volunteers were aged between 30 and 65 years, with serum TC levels less than  $6.2 \text{ mmol L}^{-1}$ , serum TG levels less than  $2.3 \text{ mmol L}^{-1}$  and a body mass index (BMI) between 25 and  $35 \text{ kg/m}^2$ . Recruitment was done via a telephone interview with eligible volunteers as well as the placement of study flyers on the clinic noticeboard. In addition, study posters and pamphlets were placed at local community centres including health and fitness centres and the local council office

Exclusion criteria were smoking; suffering from diabetes, kidney, liver, or inflammatory intestinal diseases, thyroid disorders, immunodeficiency diseases, or lactose intolerance; taking nutritional supplements within the previous three weeks or during the eight-week study period; receiving cholesterol lowering medication, estrogen, progesterone, or diuretics; being pregnant or breast feeding; and consuming probiotic yogurt or any other probiotic products within the previous 2 months.

Any dietary preferences such as vegan or vegetarian was not the main focus of the selection process for this study as the participants were mainly selected as per their serum TC and TG levels and not as per their food selections.

The sample size of each study was usually determined by the level of significance ( $\alpha$ ) and statistical power of the study. The value of  $\alpha$  is associated with the confidence level (Cl) of the test and usually reported as  $\alpha = 0.05$  and 95% Cl. The power of the study sets the level of significance that the results will be accepted as being 'significant'. A power of 80% is often chosen as raising power to 90% power which will increase the sample size by about 30% and raise it to 95% which would entail a 60% increase in sample size, substantially increasing costs of the study (Fischer et al., 2011). In addition, the primary information obtained from the most

relevant, previously reported results can be used in sample size estimation. For example, Ejtahed et al. (2011) investigated the effect of probiotics on serum TC level and reported for an expected change of 0.25 mmol L<sup>-1</sup> in TC level between intervention and control groups and by considering  $\alpha = 0.05$  and a power of 80% , the sample size was calculated as 30 per group. The study by Naruszewicz et al. (2002 ) also involved thirty-six study participants into two groups (18 per group) to determine the influence of daily consumption of 400 mL of a rose-hip drink containing *L. plantarum* 299v for six weeks on their lipid profiles.

Therefore, as per information obtained from the most relevant reported studies and considering  $\alpha = 0.05$  and a power of 80%, the sample size was computed as fifteen per group (Study Size 3.0 software). This number was increased to 16 sixteen per group to accommodate the anticipated dropout rate.

This study was approved by Victoria University Human Research Ethics Committee (HRE 13-039). Prior to the trial, a full explanation concerning the purpose and methodology of the study was given to the participants and written informed consent was obtained from all participants (Appendix 6.1).

### **6.2.2 Study design**

This study was designed as a parallel, double-blind, randomised and controlled clinical trial. Sixteen participants were randomly assigned to three groups, which matched participants in each group based on gender and age.

A pre-adjustment period of one week was designated, during which all participants had to refrain from taking yogurt or any other fermented dairy food. Over the study period of eight weeks, the participants were allocated daily yogurt samples to consume.

All participants were instructed to maintain their usual dietary habits and exercise routine, to keep the yogurt under refrigeration and avoid consuming any yogurt other than that provided

to them. The study was double-blind, which is, neither the subjects nor the researcher knew which treatment any particular subject received.

The yogurt samples (C, Syn, and SynP) were distributed weekly and subjects were reminded via phone call. Information on food consumption, anthropometric measurements and fasting blood samples (FBS) were collected at the beginning (week 1), in the middle (week 4) and at the end of the trial (week 8). Nutrient intakes were estimated using 24-h dietary recall at the beginning and at the end of the study for three days. Three-day averages of energy and macronutrient intakes were analysed using FoodWorks (Xyris 7) 4 software (First Databank Inc., Hearst Corp., San Bruno, CA, USA). If a participant ate a food that was not in the database, a food with close nutrient composition was chosen. Nutrient information was also estimated through food labels or recipes supplied by participants.

### **6.2.3 Anthropometric measurements**

Anthropometric measurement of participants included height, weight (BW), waist and hip circumference, BP and heart beat rate. Body weight was measured using a Seca medical scale (Seca, Hamburg, Germany) with 0.1kg accuracy without shoes and wearing light clothing. Heights were measured without shoes using a wall mounted Seca stadiometer (Seca, Hamburg, Germany) with 0.1cm accuracy. BMI was calculated by dividing BW (kg) by height squared ( $m^2$ ). Waist circumference was measured twice with the participants standing with their arms crossed across their thorax with a tape measure placed around the body at the narrowest point between the lower costal (10th rib) border and the iliac crest. Hip circumference was also measured twice, with the participants standing in a relaxed position with arms folded across their thorax at the level of the greatest posterior protuberance of the buttocks. BP and heart beat rate were measured twice using an electronic blood pressure monitor, where the inflatable cuff of the sphygmomanometer was positioned round the upper arm at the heart level. All

measurements were recorded on summary sheets which listed each participant's information and then saved on an excel data sheet for future analysis.

The subjects were directed to the Health Scope Blood Collection Centre (54 Child Road, Epping, Australia) at the beginning of the study, then on week 4 and at the end of week 8 for blood tests. The blood samples were drawn from the antecubital vein in the arm, after overnight (8-10 h) fasting between 7:00 and 10:00 a.m. by a qualified phlebotomist. The blood samples were labelled with the participants' code for confidentiality and stored on ice until they were centrifuged to separate the serum, which was stored at -20°C waiting analysis.

#### **6.2.4 Biochemical analysis**

Blood samples were analysed at the Health Scope Pathology Centre (1868 Dandenong Road, Clayton, 3168, VIC). Serum concentrations of TC, TG, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), LDL-C/HDL-C and TC/HDL-C ratios, and C-reactive protein (CRP) were measured using the standard enzymatic colorimetric method (Roache/Hitachi cobas c 501 analyser, Roache Diagnostics, USA). The serum TC, LDL-C, HDL-C, TG, LDL-C/HDL-C and serum TC/HDL-C ratios can be considered as atherogenic indices, whereas CRP level can be used as the inflammation marker when the serum lipid profile is elevated (WHO, 2015).

#### **6.2.5 Dietary Intervention**

All yogurt samples contained 0.02% (w/v) freeze-dried YC-380 thermophilic yogurt cultures for direct vat set (*Streptococcus thermophiles* 1275 and *Lactobacillus bulgaricus* 1842). The control samples (control) only contained standard yogurt cultures (Refer to section 5.2.2.1), whereas the synbiotic yogurt (Syn) was supplemented with 0.5% (v/v) *Lactobacillus acidophilus* ASCC 2404, *Lactobacillus rhamnosus* ASCC 1520, and 2% (w/v) Synergy 1 (Refer to section 5.2.2.2), and, the synbiotic yogurt containing pomegranate juice

concentrate (PJC) (SynP) was the same as above with 20% (v/v) PJC added to the milk before incubation (Refer to section 5.2.2.3). The yogurts were produced weekly and distributed to the participants.

The percentage of difference in mean  $\pm$  SD value of any blood parameters for each treatment group vs control was calculated as follows:

$$\% \text{ of difference: } C - T / C * 100$$

C and T represent the mean value of each blood parameter in the control and in each treatment group, respectively.

### **6.2.6 Statistical analysis**

The experimental data were analysed by SAS software (SAS Institute Inc, 2008) and the results were expressed as mean  $\pm$  standard deviation. Analysis of variance (ANOVA) was used to identify any differences among the three groups or among the three intervals within a group. Multiple comparisons were conducted by the *post hoc* Tukey test. The changes in anthropometric measurements, nutrient intakes and blood parameters of the participants throughout the trial period were compared by t-test. Differences with ( $P < 0.05$ ) were considered statistically significant.

## **6.3 RESULTS**

Microbiological analyses of yogurt samples (C, Syn, and SynP) showed that at the beginning of the study (at the point of manufacturing), the total bacterial count in C, Syn and SynP samples were  $8.45 \pm 0.21 \text{ Log}_{10} \text{ CFU g}^{-1}$ ,  $9.12 \pm 0.41 \text{ Log}_{10} \text{ CFU g}^{-1}$ ,  $9.61 \pm 0.33 \text{ Log}_{10} \text{ CFU g}^{-1}$ , respectively. As mentioned in section 6.2.6, the yogurt samples were distributed to the participants within a week post production. Therefore, the total bacterial count in C, Syn and SynP samples at the point of consumption were  $8.35 \pm 0.34 \text{ Log}_{10} \text{ CFU g}^{-1}$ ,  $9.20 \pm 0.41 \text{ Log}_{10} \text{ CFU g}^{-1}$ ,  $7.32 \pm 0.54 \text{ Log}_{10} \text{ CFU g}^{-1}$ , respectively.

The mean values  $\pm$  standard deviations of weight, height, age and BMI of the study subjects at the beginning of the study did not indicate any significant differences among the three study groups. The mean age was 47 years for Syn group, 48.5 for SynP group and 48 years for the control group.

No significant changes were detected in the subjects' weight and BMI, and their intake of total fat, SFA, and dietary fibre during the eight weeks of the study within the groups (Table 6.1). One-way analysis of variance with the *post hoc* Tukey test showed no statistically significant change in total energy and cholesterol intakes at the beginning of the study among the three groups. There was no significant difference in total energy or cholesterol intake throughout the study within a group (Table 6.1).



**Table 6.1** Anthropometric data and nutrient intake of the study participants throughout the study

Variables <sup>b</sup>	Group	W1	W4	W8	P
		Mean ± SD	Mean ± SD	Mean ± SD	
Weight (kg)	Syn <sup>c</sup>	88.51±5.8	88.40±5.9	88.01±6.2	0.96
	SynP	86.69±6.1	86.80±5.9	85.91±5.1	0.99
	C	86.30±6.6	85.34±6.9	85.12±6.8	0.95
BMI (kg/m <sup>2</sup> )	Syn	28.04±1.5	28.00±1.6	27.98±1.4	0.99
	SynP	28.40±1.7	28.40±1.7	28.22±1.5	0.93
	C	28.85±2.0	28.41±2.3	28.31±1.7	0.73
Total fat (%energy)	Syn	34.03±5.1	34.02±5.0	33.91±5.0	0.89
	SynP	35.70±5.0	35.01±5.2	34.80±3.9	0.39
	C	35.71±5.1	35.71±5.1	35.11±5.0	0.23
SFA(% energy)	Syn	11.00±1.3	10.60±1.4	10.21±1.2	0.81
	SynP	10.90±1.6	10.40±1.2	10.01±2.2	0.29
	C	11.50±1.6	10.61±1.2	10.90±1.4	0.15
Dietary fibre	Syn	25.32±4.4	25.56±4.39	26.33±4.0	0.82
	SynP	24.71±4.7	26.47±5.51	26.54±5.5	0.42
	C	25.47±5.2	26.06±4.69	26.08±4.7	0.15
Total energy (kj)	Syn	8047.00±951.8	8033.0±932.4	7876.67±874	0.39
	SynP	8232.20±1038.3	8158.33±891.6	8087.33±851.4	0.93
	C	8337.47±1124.4	8251.06±1013.8	8195.33±962.2	0.87
Cholesterol (mgL <sup>-1</sup> )	Syn	229.15±47.3	227.30±47.3	218.85±43.2	0.61
	SynP	242.61±36.9	242.90±36.9	237.18±35.9	0.38
	C	235.66±47.6	234.86±47.6	229.11±46.1	0.52

<sup>a</sup> W1, data collection at the beginning of the study; W4, data collection after 4 weeks; W8, data collection after week 8

<sup>b</sup> Data are presented as means ± SD. Differences with  $P < 0.05$  were considered to be statistically significant

<sup>c</sup> Group Syn yogurt sample supplemented with *Lactobacillus acidophilus* ASCC 2404, *Lactobacillus rhamnosus* ASCC 1520, 2% Synergy 1, Group SynP: yogurt sample enriched with *Lactobacillus acidophilus* ASCC 2404, *Lactobacillus rhamnosus* ASCC 1520, 2% Synergy 1 and 20% PJC and Group C: yogurt sample with standard yogurt cultures only.

No statistically significant difference was observed among the three groups in blood parameters at baseline. Neither was there any statistically significant difference throughout the study for blood parameters within the C and Syn groups; however, in the SynP group, there was a significant drop (18.75%) in TC: HDL-C ratio ( $P < 0.05$ ). In addition, comparing the differences between baseline (W1), week 4 (W4), and week 8 (W8) for TG and HDL-C levels has also shown no statistically significant difference among the C and Syn groups; however, in the SynP group, TG level was significantly reduced by 9.81% ( $P < 0.05$ ) at the end of the study (Table 6.2). The *post hoc* test showed that the decrease in TC and LDL-C was significant for

both the Syn and SynP groups ( $P < 0.001$  for both) compared with the control group ( $P = 0.006$  and  $P = 0.001$ , respectively). Consumption of the synbiotic yogurt containing PJC (SynP) resulted in a 19.0% decrease in TC and a 23.0% decrease in LDL-C levels compared with the controls, whereas in the Syn group, TC and LDL-C levels were reduced by 14.0% and 17.0%, respectively at the end of the study (Figs 6.1 and 6.2).

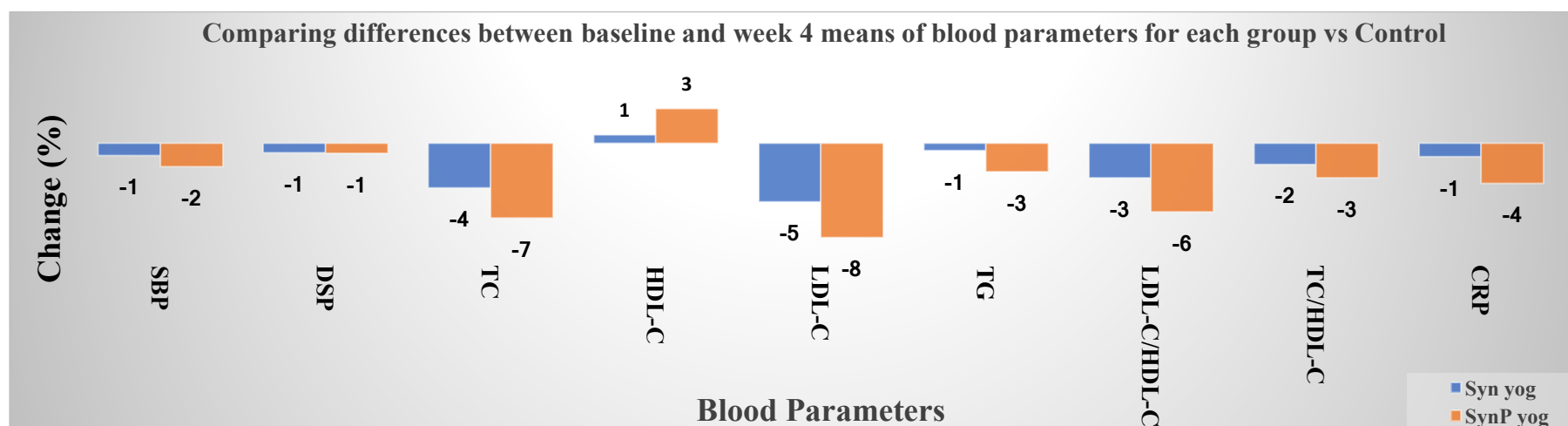
**Table 6.2** Trends in blood parameters of the participants during the study (W1,W4 &W8) <sup>a,b,c</sup>

Variables <sup>b</sup>	Groups	W1	W4	W8	<i>P</i>
		Mean ± SD	Mean ± SD	Mean ± SD	
SBP	Syn <sup>c</sup>	125.93±4.9	123.9±3.35	122.67±3.01	0.07
	SynP	123.93±4.9	122.6±3.35	121.6±3.01	0.06
	C	127.07±6.3	125.2±5.7	124.27±4.8	0.38
DBP	Syn	83.00±1.88	82.1±1.64	81.87±1.55	0.17
	SynP	82.8±1.88	82.1±1.64	82.13±1.72	0.28
	C	83.47±2.3	82.8±2.07	82.27±2.01	0.31
TC	Syn	6.00±0.3	5.63±0.2	5.22±0.3	0.006
	SynP	6.66±0.2	5.80±0.2	5.23±0.3	<0.001
	C	6.39±0.6	6.12±0.62	6.03±0.6	0.28
HDL-C	Syn	1.32±0.3	1.24±0.3	1.27±0.3	0.72
	SynP	1.5±0.2	1.40±0.3	1.35±0.3	0.61
	C	1.27±0.1	1.27±0.1	1.25±0.2	0.91
LDL-C	Syn	3.64±0.4	3.44±0.4	2.98±0.3	0.009
	SynP	4.26±0.4	3.70±0.4	3.27±0.2	0.002
	C	4.39±0.6	4.06±0.7	3.91±0.7	0.14
TG	Syn	1.58±0.8	1.50±0.6	1.23±0.4	0.030
	SynP	2.2±0.9	1.87±0.6	1.64±0.5	0.04
	C	1.57±0.7	1.57±0.6	1.56±0.5	0.75
LDL-C/HDL-C	Syn	1.59±0.7	1.56±0.8	1.43±0.6	0.22
	SynP	1.63±0.7	1.53±0.8	1.42±0.6	0.18
	C	3.45±0.4	3.20±0.4	3.22±0.54	0.30
TC/HDL-C	Syn	4.67±0.9	4.43±1.1	4.27±0.9	0.03
	SynP	4.83±0.9	4.30±1.1	3.96±0.9	0.15
	C	4.95±0.5	4.80±0.5	4.89±0.8	0.86
CRP	Syn	3.89±2.13	3.47±1.9	3.21±1.8	0.63
	SynP	3.31±2.11	2.90±1.9	2.78±1.8	0.73
	C	5.47±4.67	5.03±4.2	4.99±4.3	0.94

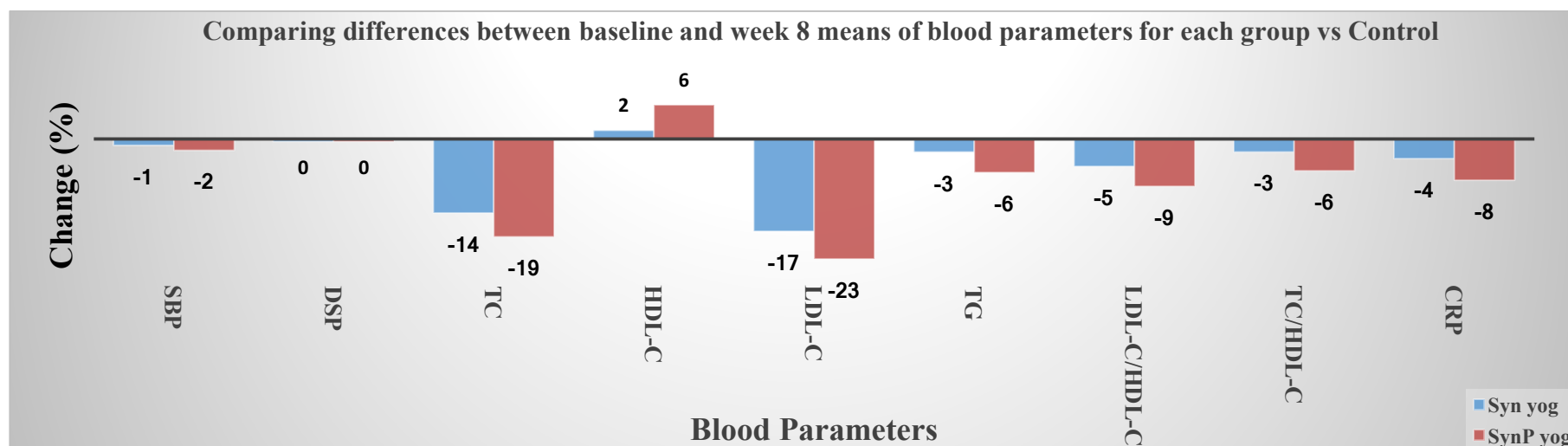
<sup>a</sup> T1, data collection at the beginning of the study; T2, data collection after 4 weeks; T3, data collection after week 8

<sup>b</sup> Data are presented as means ± SD. Differences with *P* < 0.05 were considered to be statistically significant

<sup>c</sup> Group Syn: yogurt sample supplemented with *Lactobacillus acidophilus* ASCC 2404, *Lactobacillus rhamnosus* ASCC 1520, 2% fructooligosaccharide enriched inulin (FEI), Group SynP: yogurt sample enriched with *Lactobacillus acidophilus* ASCC 2404, *Lactobacillus rhamnosus* ASCC 1520, 2% FEI and 20% PJC and Group C: yogurt sample with standard yogurt cultures only.



**Figure 6.1** Comparing differences in mean value of blood lipid parameters (each group vs control) at the end of week 4



**Figure 6.2** Comparing differences in mean value of blood lipid parameters (each group vs control) at the end of week 8

## 6.4 DISCUSSION

The promotion of a healthy lifestyle using non-pharmacological means is a preventive strategy aiming to improve lipid profile and therefore, reduce a substantial number of deaths from CVD, particularly heart attacks and strokes (Hussein et al., 2014; WHO, 2015). In addition to using probiotics and prebiotics to alleviate hypercholesterolemia and consequently improve blood pressure (BP) at the population level, certain foods containing phytochemicals (e.g. antioxidants and polyphenols) can also be used to have a similar impact by altering the body metabolism (Juurlink, 2003).

In the present study, weight, BMI, energy, and nutrient intakes of the participants within any three groups; did not significantly change during the study. Therefore, the observed decrease in serum TC and LDL-C concentrations in Syn and SynP groups could not be due to the changes in weight and dietary intakes.

Although we did not observe any statistically significant changes in nutrient intakes during the course of the study, there was a non-significant reduction ( $P > 0.05$ ) in daily energy and SFA intakes in all groups. At the beginning of the study, the participants were asked to record their food intake, therefore, they might have changed their eating habits while keeping the food records. One of the most frequently given reasons for this change was to lessen the inconvenience of recording food intake (Rebro et al., 1998).

Within all probiotic strains investigated for cholesterol-lowering effect, *L. acidophilus* has been the most studied strain. Anderson and Gilliland (1999) reported that daily consumption of 200 g of yogurt containing *L. acidophilus* L1 contributed to a 2.9% decrease in serum cholesterol concentration in hypercholesterolemic humans. In a study by Ataie-Jafari et al. (2009), a significant decrease in serum TC was observed with the daily consumption of 200g of probiotic yogurt containing *L. acidophilus* and *B. lactis* in

hypercholesterolemic people. Sadrzadeh-Yeganeh et al. (2010) has also demonstrated that daily intake of 300 g of probiotic yogurt containing *L. acidophilus* La5 and *B. lactis* Bb12 had positive effects on the lipid profile of healthy women. These effects include a decrease in TC in both conventional ( $P < 0.05$ ) and probiotic groups ( $P < 0.005$ ) as well as a drop in TC: HDL-C ratio for conventional ( $P < 0.05$ ) and probiotic groups ( $P < 0.001$ ) compared with the control group, and an increase in HDL-cholesterol in the probiotic yoghurt group ( $P < 0.05$ ) compared with the control group.

Furthermore, studies that used probiotic capsules instead of dairy products for administering probiotics did not support the cholesterol-lowering potential of probiotics (Lewis & Burmeister, 2005; Greany et al., 2008). It was proposed that dairy products are a more effective vehicle for administering probiotics. Lewis and Burmeister (2005) postulated that sufficient time was not available for the freeze-dried bacteria in probiotic capsules to become metabolically active in the intestine before being flushed into the colon. These inconsistent findings could be partly because of varying strains and doses of probiotics, different duration of treatment periods, sample size, and variations in clinical characteristics of participants (Ooi & Liong, 2010).

In addition, the hypocholesterolaemic effect observed in Syn and SynP groups could be due to the incorporation of synergy1 in synbiotic yogurt production. It has been proposed that when probiotics settle in the gut, they could ferment indigestible food ingredients including prebiotics and raise the amount of short chain fatty acids (SCFA) in the gut. These large molecules are depolymerised by a variety of hydrolytic enzymes produced by the gut microbiota and they are able to lower the blood serum lipids through blocking the synthesis of hepatic cholesterol and/or through redirecting plasma cholesterol towards the liver (Kim & Shin, 1998; Wolever et al., 2002; Xiong et al., 2004). By producing bile acids through deconjugating of the bile salts in the small intestine, probiotics prevent micelle production.

When cholesterol enters the enterohepatic circulation, it is dealt with in the same way. Probiotics, by using hydroxy steroid dehydrogenase and conjugated bile acid hydrolase enzymes, break down the bile acid and hydrolyse bile salts. By doing so, the enterohepatic circulation of bile acids will be disrupted (Gunnessa & Gidley, 2010; Lye et al., 2010). In addition, probiotic bacteria reduce the absorption of cholesterol in the intestine by binding and possibly incorporating onto the cell membrane. Cholesterol can also be assimilated during probiotics growth. However, for these mechanisms to operate, lactic acid bacteria (LAB) must be alive when passing through the GIT.

Although the effect of PJ on ameliorating the plasma lipid profile parameters has been reported in the literature, a decisive outcome cannot be reached due to the controversies raised in this area. Yatsunenko et al. (2012) documented that the complex interaction between pomegranate polyphenols such as ellagitannins and microbiota in the intestinal environment could be due to the total count of gut bacteria. Accordingly, the punicalagins and ellagitannins are transformed by human gut bacteria to specific metabolites called dibenzo-pyranone-type urolithins which possess antioxidant properties; while the remaining punicalagins and ellagitannins remain unabsorbed in the gut lumen. Le Chatelier et al. (2013) reported that individuals with a low gut microflora are more likely at risk of developing dyslipidaemia compared to those with high gut bacterial counts.

Bialonska et al. (2010) have shown that any PJ with at least 1000-2000 mgL<sup>-1</sup> gallic acid equivalent (GAE) phenolic compounds can be considered as a standardized dietary supplement to increase the daily antioxidant intake in any clinical study. Our study is the first randomised controlled clinical trial investigating the effect of synbiotic yogurt supplemented with pomegranate polyphenols on the lipid profile and BP of hypercholesterolemic subjects. Our findings agree with the findings of an animal study undertaken Sakr and Abou Dawood (2015) reporting that yogurt supplemented with either

PJ or pomegranate peels water extract had a good cholesterol-lowering effect in hypercholesterolemic rats.

The pomegranate juice concentrate (PJC) used in our study was made from Wonderful pomegranate variety, previously evaluated for its physicochemical and phytochemical properties by our research team Arjmand (2011) and contained *ca.* 1590 mgL<sup>-1</sup> GAE phenolic compounds.

Although the present results showed no statistically significant difference in SBP and DBP within any group; however, there was a significant drop in TC: HDL-C ratio and TG levels in both Syn and SynP groups when they are compared with the control group ( $P < 0.05$ ). At baseline, the SFA: total fat ratio was 0.32 for both the Syn and control groups and 0.31 for the SynP group. The ratio decreased to 0.31, 0.30, and 0.29 for the control, Syn, and SynP groups, respectively. This drop could be due to the reduction in SFA and total fat intakes throughout the study; however, it was not significant ( $P > 0.05$ ). In addition to these changes, there was a significant decrease in the mean difference of TC and LDL-C levels in both Syn and SynP groups compared with the control ( $P < 0.001$ ). This reduction could be the consequence of probiotic activity in the gut after consuming synbiotic yogurt for eight weeks. However, our results also showed a better reduction in TC and LDL-C levels for the SynP group, indicating the incorporation of PJC in yogurt resulted in a better hypocholesterolaemic impact compared with other groups.

*In vitro* hypotensive properties of the synbiotic yogurt with 20% PJC was confirmed in Chapter 5 ( Refer to section 5.3.4.6); however, it did not have any impact on the blood pressure of participants throughout the study period. This could be due to the fact that PJC supplementation would have hypotensive effects; however, it may require a longer design study.



The limitations of this study included its short duration and small sample size. Further investigations with longer duration and a no-yogurt control group are needed for better confirmation of the positive effects of probiotic yogurt on the lipid profile in hypercholesterolaemic people.

## **6.5 CONCLUSION**

In conclusion, this study showed that consumption of synbiotic yogurt containing 0.5% (v/v) *L. acidophilus* ASCC 2404 and *L. rhamnosus* ASCC 1520, and 2% (w/v) Synergy 1 could decrease serum TC and LDL-C concentrations in hypercholesterolaemic people. According to our results, we can also conclude the promising effect of PJC as a food supplement, especially when combined with probiotics and prebiotics in fermented dairy products to deliver its hypocholesterolemic effect. However, well-designed and long-term studies are required to investigate the hypotensive impact of PJC supplemented-dairy foods.

## **7. OVERALL CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS**

The results of probiotic evaluation for acid and bile tolerance, cholesterol removal ability and hypotensive properties showed that all strains of *Lactobacillus* and *Bifidobacteria* studied were able to tolerate pH 2.0 for 2 hours despite variations in the degree of viability. *L. acidophilus* CSCC 2413, *L. zeae* ASCC 15820, *B. longum* CSCC 5022, *L. acidophilus* CSCC 2404 were the most acid-tolerant strains, with more than  $10^7$  CFU mL<sup>-1</sup> after 2h incubation at pH 2.0, while *L. plantarum* 276, *L. acidophilus* CSCC 2400 and *L. paracasai* ASCC 279 were the most acid-sensitive strains, with only  $10^4$  CFU mL<sup>-1</sup> after 2h incubation.

All selected strains could tolerate the presence of bile salts, with greater tolerance towards oxgall than taurocholic acid. Different growth rates observed in the presence of selected bile salts suggested that taurocholic acid provided more growth suppression towards probiotics than oxgall. The most bile resistant strains were identified as *L. rhamnosus* ASCC 2607, *acidophilus* CSCC 2404, and *L. rhamnosus* ASCC 1520.

The ACE inhibition effect was significantly different among the strains studied and ranged from a maximum of 78.5% to a minimum of 8.2% inhibition. Strains *L. zeae* ASCC 15820, *L. rhamnosus* ASCC 1520, *B. bifidum* CSCC 5286 and *B. longum* CSCC 5089 provided the highest ACE-I activity among the selected strains (78.5, 76.9, 73.8 and 72.2%, respectively), followed by *L. acidophilus* CSCC 2404, which showed a relatively reasonable antihypertensive property (60%).

All strains studied possessed varying degrees of cholesterol removal capabilities from the growth medium through several mechanisms, including cholesterol assimilation, or incorporation of cholesterol into the cellular structure, or binding of cholesterol onto the cellular membrane and/or bile salt deconjugation, and bile salt hydrolase activity (BSH). Results of fatty acid analysis of harvested probiotic cells and bile salt deconjugation studies showed that the cholesterol incorporation and binding to the cell membrane, and deconjugation were the most effective mechanisms. Strains of *L. zeae* ASCC 15820, *L.*

*acidophilus* CSCC 2410, *L. rhamnosus* ASCC 2607, and *L. paracasai* ASCC 279 showed higher deconjugating activity in the presence of sodium glycocholate than sodium taurocholate and the mixture of bile salts. Substrate preference for BSH was more towards sodium glycocholate than sodium taurocholate. *L. acidophilus* CSCC 2400 and *B. longum* CSCC 5022 exhibited higher bile salt hydrolysis specific activity towards all bile salts than other strains. Most strains of *Lactobacillus* and *Bifidobacterium* exhibited higher total BSH activity on bile mixtures compared to when bile salts were used individually.

Among all probiotics studied, *B. longum* CSCC 5022, *L. acidophilus* CSCC 2404, *L. acidophilus* CSCC 2410 and *B. bifidum* CSCC 5286 demonstrated higher cholesterol removal ability. The pattern of cholesterol removal showed similarities to that of bacterial growth, indicating that cholesterol removal was associated with growth. However, the cholesterol removal by resting and dead cells confirmed that even non-viable cells of these probiotics still had the ability to bind the cholesterol, and therefore could be used as cholesterol-reducing agents in the gastrointestinal system.

In terms of the prebiotic selection, the microbiological and physicochemical evaluations showed that addition of 2.0% (w/v) Synergy 1 to yogurt milk resulted in optimal probiotic growth leading to maximum ACE-I inhibition activity in the synbiotic yogurt.

The most effective concentration of pomegranate juice concentrate (PJC) with the optimal hypotensive properties over the 21-day storage period was found to be 20% supplemented post-heat treatment, and no apparent antagonism was observed between the cultures' activity and the PJC phytochemicals during the incubation time. Further analyses of the synbiotic yogurt supplemented with PJC indicated that, compared to the control yogurt, it had a higher colour index and contained 63% more TPC, 94% more AA and 75% more ACE inhibition activity, respectively. Therefore, the synbiotic yogurt supplemented with PJC had higher health promoting properties.

The sensory evaluation of this product revealed that by increasing the PJC level, the overall acceptability among the panellists increased significantly and therefore, this product could offer a pleasant and effective route to increasing the intake of bioactive compounds and probiotics in our daily diet. Despite the slight adverse effect of PJC supplementation at a level of 20% level on probiotic numbers, the probiotic population at the end of the 21-day storage period at 4°C was still within the acceptable range ( $> 10^6$  CFU mL<sup>-1</sup>) to deliver health-promoting properties.

The result of the human study confirmed the effectiveness of a daily intake of synbiotic yogurt containing 0.5% (v/v) *L. acidophilus* ASCC 2404 and *L. rhamnosus* ASCC 1520, and 2% (w/v) synergy1 in reducing the serum TC and LDL-C concentrations in hypercholesterolemic people. However, large-scale longitudinal studies are required to establish the hypotensive impact of the developed product with hypercholesterolemic people as has been confirmed in *in-vivo* studies.

There are a number of criteria that should receive more attention in future research studies as listed here:

1. To overcome the textural problems of the developed synbiotic yogurt with PJC, more investigation is needed into the formulation and processing of yogurt (such as using sodium caseinate or carrageenan in yogurt formulation).
2. Large-scale longitudinal studies are required to substantiate the hypercholesterolemic and hypotensive properties of the PJC-supplemented synbiotic yogurt developed in this project
3. The next phase of this study would involve developing a product-commercialisation plan and working with an industry partner to make the product available on the market.

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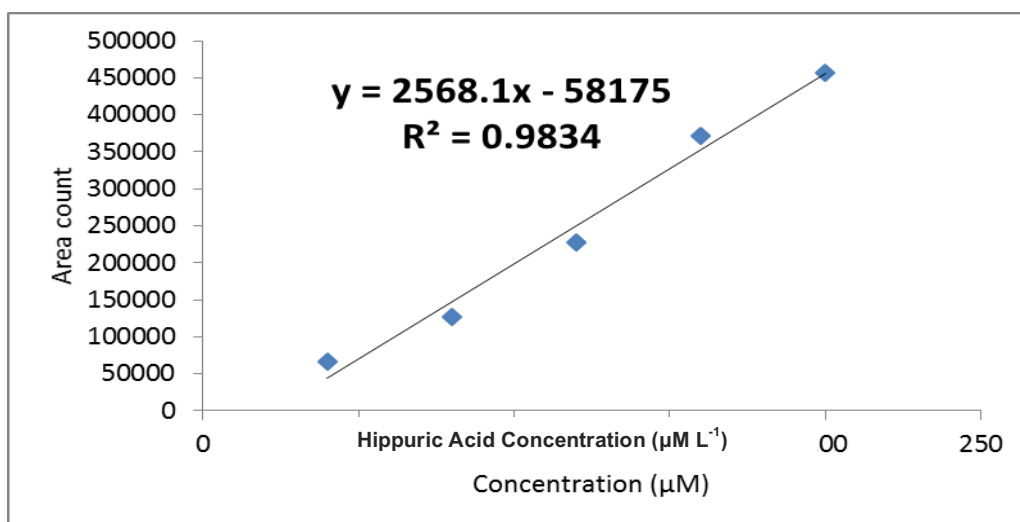
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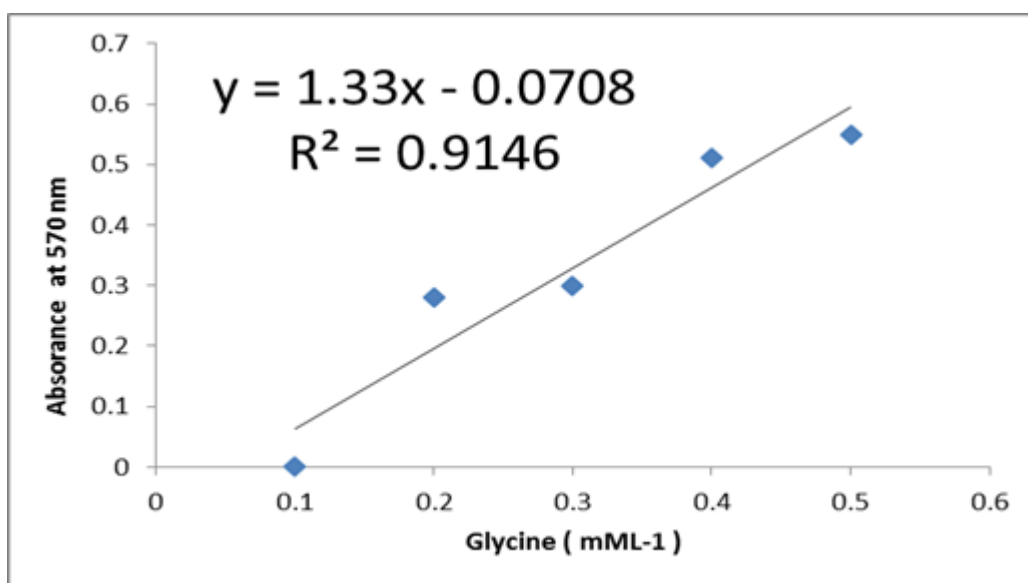
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## **Appendixes**

### Appendix 3.1



### Appendix 4.1



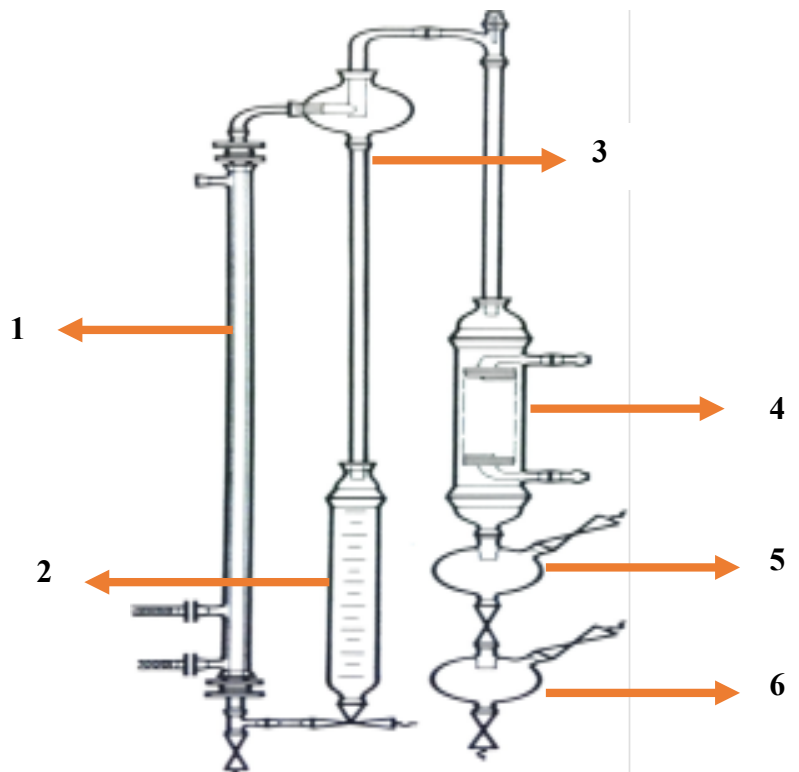
## Appendix 5.1

### Climbing Film Evaporator

Pomegranate juice was concentrated to 52 °B using a Climbing Film Evaporator (Jobling, James A Jobling and Co Ltd, Stoke-on-Trent, UK). The unit has been designed for the concentration of heat sensitive materials under strong vacuum (-85 kPa). It includes a calandria tube 3 meters long with 25 mm nominal bore and a light wall glass heat exchange tube enclosed in a steam jacket. The vapours and liquid being concentrated rise through the inner glass tube and are separated in a separator on the top of the unit. The vapours pass into a condenser and then into two receiver vessels. The concentrate is collected in a graduated receiver and is recirculated via a three-way valve until the target level of concentration is reached, when it can be removed for packaging and storage.

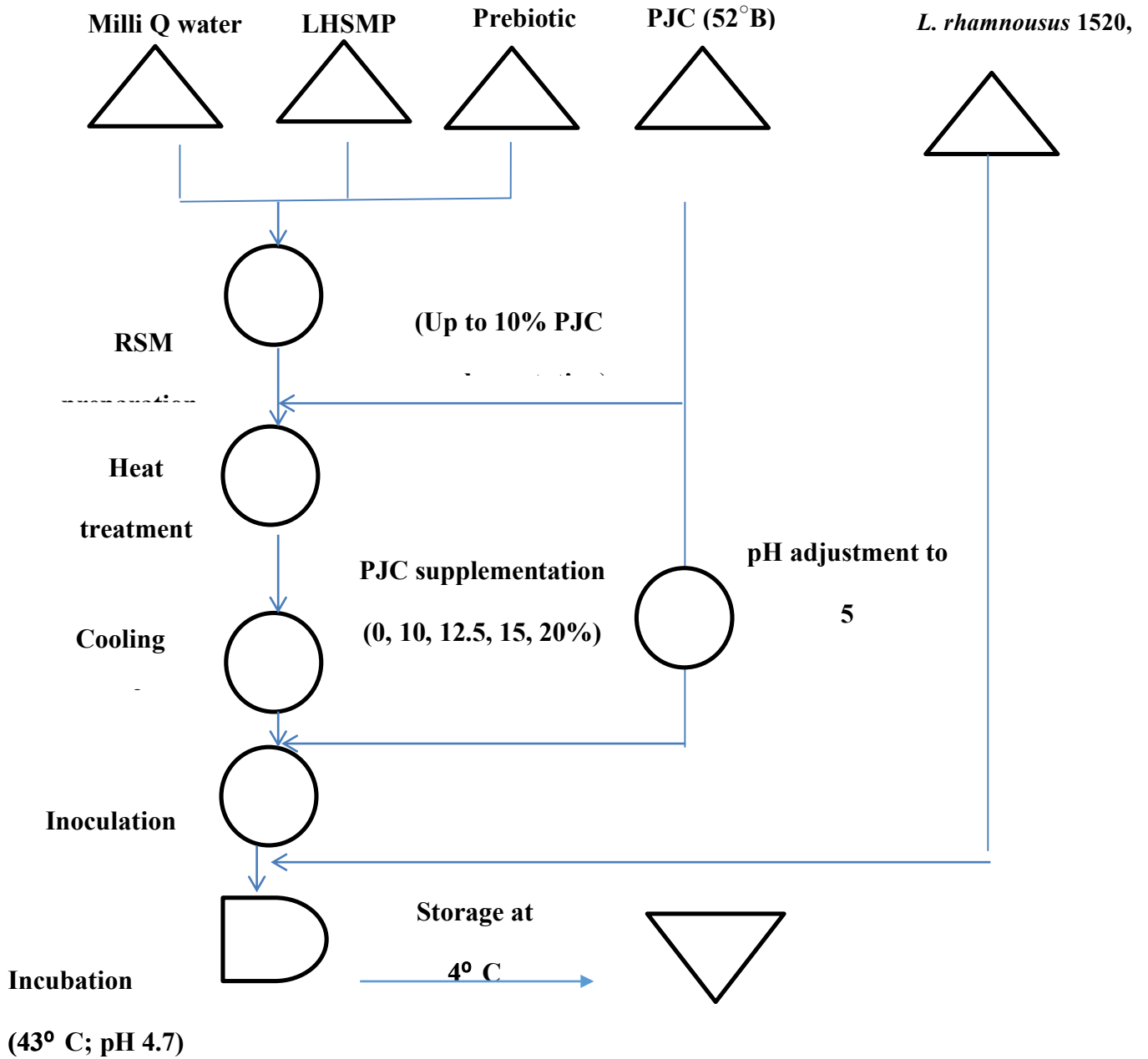
### Climbing Film Evaporator

1. Calandria,
2. Concentrate receiver,
3. Separator,



## Appendix 5.2

Process flow diagram of yogurt production supplemented with PJC (52<sup>0</sup>B)





## Appendix 5.3

### Questionnaire for sensory evaluation of yogurt samples

#### Instruction:

You are given three different yogurt samples (flavoured and non-flavoured), coded 124, 781, and 352. Please evaluate them on the ten -point hedonic scale as below:

Remove the lid of the cups and evaluate aroma first and then the colour and appearance by visual observation. For textural properties break down the yogurt gel with spoon and gently mix the samples to evaluate the yogurt thickness. After placing product in your mouth evaluate the gel firmness and flavour.

	Aroma									
	Plain Yogurt					Fruity Yogurt				
781	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10
124	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10
352	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

	Colour									
	White			Light Pink				Deep Pink		
781	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10
124	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10
352	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

### Appearance

**Very grainy**

**Not grainy**

<b>781</b>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

<b>124</b>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

<b>352</b>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

**Gel thickness:** To what extend is easy to mix the samples with spoon

**Thin**

**Thick**

<b>781</b>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

<b>124</b>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

<b>352</b>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

**Gel firmness:** The force required to compress sample between tongue and palate

**Very Soft**

**Firm**

<b>781</b>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

<b>124</b>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

<b>352</b>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

**Flavour:** Fruity sensation

	Weak fruity					Strong fruity				
781	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10
124	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10
352	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

**Overall acceptability:** Please indicate how much you like each sample

	Like least					Like most				
781	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10
124	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10
352	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
	1	2	3	4	5	6	7	8	9	10

**Additional comments on aroma flavour or texture of samples**

## **CONSENT FORM FOR PARTICIPANTS INVOLVED IN RESEARCH (sensory evaluation)**

### **INFORMATION TO PARTICIPANTS:**

We would like to invite you to evaluate the sensory attributes of our developed 124biotic yogurt. This study is the first part of our clinical study titled **“The effects of 124biotic yoghurt on lipid profile in mildly to moderately hypercholesterolemic men and women”**, conducted by the collage of Health and Biomedicine, Victoria University, St Albans as part of a PhD Research Project belonging to a registered dietician and student researcher Fatemeh Miremadi.

The study aims to investigate the effects of daily consumption of symbiotic yoghurt on the lipid profile in people with mild to moderate hypercholesteremia (cholesterol level less than 6.2 mmol/L and triglyceride level less than 2.3 mmol/L), over 8 weeks. We will be measuring height, weight, waist and hip circumference, blood pressure and taking blood samples over approximately 8 weeks at three time points (beginning of study, week 4 and at the end of study, week 8) to measure serum concentration of total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG). Symbiotic yoghurt is a mixture of a probiotic (beneficial microorganisms) and a prebiotic (carbohydrate) that are associated with favourable health effects. Probiotic microorganism has the ability of cholesterol assimilation that may reduce the serum concentration of LDL cholesterol and triglycerides, and increase the beneficial HDL level; however the effects of consuming symbiotic yoghurts compared with regular yoghurts are unclear.

Participants will need to be men and women aged between 30 and 65, and considered overweight or have obesity, by either Body Mass Index (BMI) of between 25 and 35 kg/m<sup>2</sup>, or have a waist circumference of greater than 80 cm, and meet the exclusion criteria specified in the “Information to Participants” form.

Risks involved in the project are considered minimal, however there are some risks associated which are also detailed in the 'Information to Participants' form.

#### **CERTIFICATION BY SUBJECT**

I,

of

certify that I am at least 18 years old\* and that I am voluntarily giving my consent to participate in evaluation of study: sensory attributes of our developed 124biotic yogurt. This study is the first part of our clinical study titled **“The effects of symbiotic yoghurt on lipid profile in mildly to moderately hypercholesterolemic men and women”**,

**“The effects of 124biotic yoghurt on lipid profile in mildly to moderately hypercholesterolemic men and women”** being conducted at Victoria University overseen by Professor Lily Stojanovska and A/Professor Michael Mathai.

I certify that the objectives of the study, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by the PhD student researcher, Fatemeh Miremadi, and that I freely consent to participation involving the below mentioned procedures:

- Keeping a 3 day food recall at the beginning and end of the study
- Fasting overnight for between 8-10 hours at the beginning of the trial, week 4 and at the end of study, week 8 for the purpose of blood taking
- Daily consumption of either ordinary or symbiotic yoghurts over the study period
- Attend the College of Health and Biomedicine, Nutritional Therapy Clinic at Victoria University, St Albans campus for the purposes of blood samples and anthropometric measurements on 3 occasions. Blood samples to be taken for biochemical analysis. On each occasion, anthropometric measurements of body weight, height, waist and hip circumference and blood pressure will be taken.

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

I have been informed that the information I provide will be kept confidential.

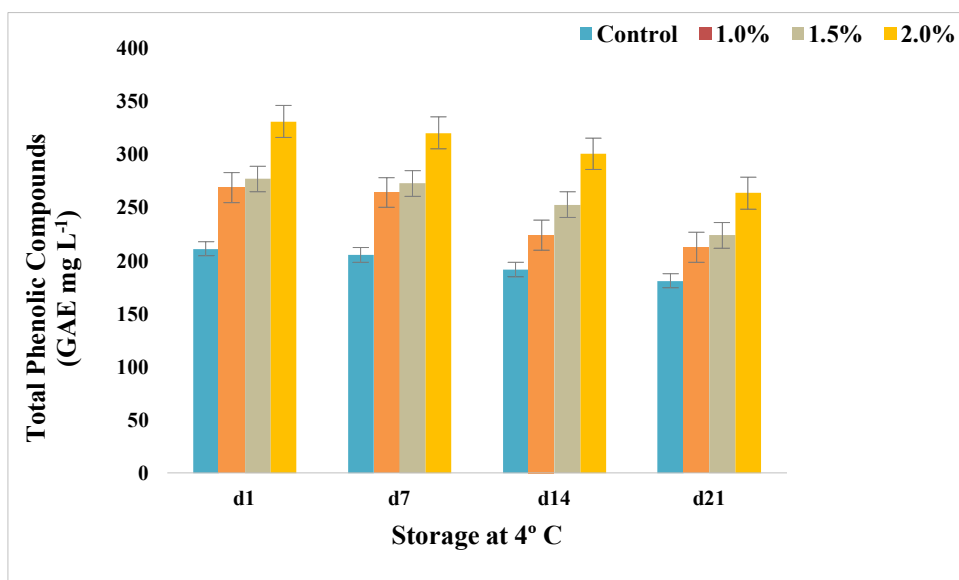
Signed:

Date:

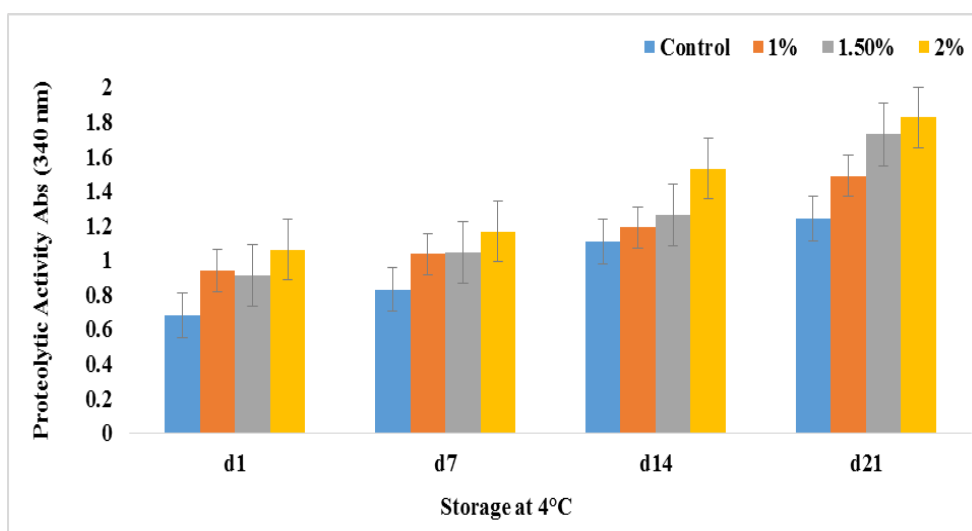
Any queries about your participation in this project may be directed to the Research Team:

1. Professor Lily Stojanovska, Principal Researcher, College of Health and Biomedicine, Victoria University, Ph: 9919 2737.
2. Associate Professor Michael Mathai, Associate Investigator, College of Health and Biomedicine, Victoria University, Ph: 9919 2211
3. Mrs Fatemeh Miremadi, PhD Research Student, College of Health and Biomedicine, Victoria University, Ph: 0448 355 535

## Appendix 5.5



**Figure 1:** The effect of supplementation with different concentrations of 124ergy1 on the phenolic compounds level in 124biotic yogurt during 21-day storage at 4°C.



**Figure 2 :** The effect of supplementation with different concentrations of 124ergy 1 on proteolytic activity of *L. rhamnosus* ASCC 1520 and *L. acidophilus* CSCC 2404 in 124biotic yogurts during 21-day storage at 4°C.

**Table 1:** Scavenging effects of yogurt samples supplemented with different concentration levels of 124ergy 1on the 1, 1-diphenyl-2-picrylhydrazyl radical

	<b>DPPH Inhibition (%)</b>			
<b>% 124.1 addition</b> <b>Storage (days)</b>	0% (Control)	1.0 %	1.50 %	2.0 %
<b>d1</b>	61.5±0.02 <sup>a</sup>	65.2±0.11 <sup>a</sup>	70.0± 0.06 <sup>a</sup>	76.5±0.30 <sup>b</sup>
<b>d7</b>	62.3±0.02 <sup>a</sup>	66.0±0.19 <sup>a</sup>	70.8± 00.06 <sup>a</sup>	77.8±0.13 <sup>b</sup>
<b>d14</b>	63.0±0.06 <sup>a</sup>	67.4±0.06 <sup>a</sup>	71.1± 0.03 <sup>a</sup>	78.1±0.07 <sup>b</sup>
<b>d21</b>	63.2±0.01 <sup>a</sup>	67.0±0.03 <sup>a</sup>	71.8±0.01 <sup>a</sup>	78.4±0.01 <sup>b</sup>

Data represents the mean ± SD of triplicate analyses from each trial. The small letter superscripts depict the statistical difference within each column,  $p < 0.05$  between means for the same yogurt batches at different time intervals.



## Appendix 6.1

### 1) Victoria University Human Research Ethics Committee Approval (HRE 13-039)

#### Quest Ethics Notification - Amendment Request Process Finalised - Application Approved

Quest.Noreply@vu.edu.au Thu 5/06/2014, 1:50 AM

[lily.stojanovska@vu.edu.au](mailto:lily.stojanovska@vu.edu.au); [Michael.Mathai@vu.edu.au](mailto:Michael.Mathai@vu.edu.au);  
[frank.sherkat@rmit.edu.au](mailto:frank.sherkat@rmit.edu.au) : [fatemeh.miremadi@live.vu.edu.au](mailto:fatemeh.miremadi@live.vu.edu.au)

Dear PROF LILIAN STOJANOVSKA,

Your amendment request for the following ethics application has been formally reviewed and finalised. »

Application ID: HRE13-039 »

Chief Investigator: PROF LILIAN STOJANOVSKA »

Other Investigators: MRS FATEMEH MIREMADI, DR FRANK SHERKAT, DR MICHAEL MATHAI, DR HENRY BUTT »

Application Title: The effects of symbiotic yoghurt on lipid profile in mildly to moderately hypercholesterolemic men and women »

The amendment request for this ethics application has been accepted and deemed to meet the requirements of the National Health and Medical Research Council (NHMRC) 'National Statement on Ethical Conduct in Human Research (2007)' by the Victoria University Human Research Ethics Committee. Approval has been granted for two (2) years from the original approval date; 29/08/2013.

Continued approval of this research project by the Victoria University Human Research Ethics Committee (VUHREC) is conditional upon the provision of a report within 12 months of the above approval date or upon the completion of the project (if earlier). A report proforma may be downloaded from the Office for Research website at: <http://research.vu.edu.au/hrec.php>. Please note that the Human Research Ethics Committee must be informed of the following: any changes to the approved research protocol, project timelines, any serious events or adverse and/or unforeseen events that may affect continued ethical acceptability of the project. In these unlikely events, researchers must immediately cease all data collection until the Committee has approved the changes. Researchers are also reminded of the need to notify the approving HREC of changes to personnel in research projects via a request for a minor amendment. It should also be noted that it is the Chief Investigators' responsibility to ensure the research project is conducted in line with the recommendations outlined in the National Health and Medical Research Council (NHMRC) 'National Statement on Ethical Conduct in Human Research (2007).'

On behalf of the Committee, I wish you all the best for the conduct of the project.

Secretary, Human Research Ethics Committee Phone: 9919 4781 or 9919 4461 Email: [researchethics@vu.edu.au](mailto:researchethics@vu.edu.au)

# Flyer Cover Letter

DATE

To the Practice Manager

Dear Sir/Madam,

I am a PhD research student from Victoria University and I am writing to seek permission to leave a flyer (attached) at your clinic with a view to recruiting potential participants for a study titled

***The effects of 124biotic yoghurt on lipid profile in mildly to moderately hypercholesterolemic men and women.***

The study is being conducted under the supervision of Professor Lily Stojanovska and A/Professor Michael Mathai from College of Health and Biomedicine, Victoria University.

The study is going to be assessed by the Victoria University Human Research Ethics Committee and part of the recruitment process is by way of a flyer at medical centres.

Should you require any further information I would be happy to provide a detailed study description.

Many thanks for your consideration, and if you have any other queries, please do not hesitate to contact me.

Yours sincerely,  
Fatemeh Miremadi  
BSc,MNutDiet (Accredited Practising Dietitian)  
PhD Candidate  
College of Health and Biomedicine

Victoria University  
PO Box 14428  
Melbourne, Vic 8001  
Work: 9919 8109  
Mob: 0437 223 646  
Email: fatemeh.miremadi@live.vu.edu.au

## Who is conducting the study?

### 8.1.1.1.1 Victoria University

#### Chief Investigator:-

Professor Lily Stojanovska MSc, PhD  
College of Health and Biomedicine  
Victoria University  
PO Box 14428  
Melbourne, Vic 8001  
Phone +61 3 9919 2737  
Fax 61 +3 9919 2465  
Email. lily.stojanovska@vu.edu.au

#### Associate Investigator:-

A/Professor Michael Mathai BSc, PhD  
College of Health and Biomedicine  
Victoria University  
PO Box 14428  
Melbourne, Vic 8001  
Phone +61 3 9919 2211  
Fax 61 +3 9919 2465  
Email. michael.mathai@vu.edu.au

#### Student Researcher:-

Fatemeh Miremadi  
BSc(Nutrition Sciences), MNutDiet  
PhD Candidate  
College of Health and Biomedicine  
Victoria University  
PO Box 14428  
Melbourne, Vic 8001  
Wk: 9919 8109  
Mob: 0437 223 646  
Email :fatemeh.miremadi@live.vu.edu.au

Any queries about participation in this project may be directed to the Principal Researcher listed above.  
If you have any queries or complaints about the way you have been treated, you may contact the Ethics and Biosafety Coordinator, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 phone (03) 9919 4148.



# EAT & ENJOY OUR SUPER YOGURT!



## PARTICIPANTS WANTED FOR A PhD RESEARCH STUDY ON THE HEALTH EFFECTS OF SYNBIOTIC YOGURT

If you meet these criteria ...

- ✓ Male or Female aged between 30-65years old;
- ✓ Are overweight or have obesity;
- ✓ Free from diabetes, anaemia, liver, kidney or heart disease ;
- ✓ Non-smoking, not pregnant or lactating;
- ✓ Free from allergies to dairy or lactose;
- ✓ Willing to have yogurt everyday over 8 weeks ;
- ✓ Can visit Victoria University, ST Albans;
- ✓ Willing to have blood samples taken on 3 occasions.

## YOU MAY BE ELIGIBLE TO TAKE PART IN THIS STUDY

For further information, please contact  
FATEMEH MIREMADI [fatemeh.miremadi@live.vu.edu.au](mailto:fatemeh.miremadi@live.vu.edu.au)  
or 0437 223 646

Super Yoghurt study 043722 3646 <a href="mailto:fatemeh.miremadi@live.vu.edu.au">fatemeh.miremadi@live.vu.edu.au</a>	Super Yoghurt study 043722 3646 <a href="mailto:fatemeh.miremadi@live.vu.edu.au">fatemeh.miremadi@live.vu.edu.au</a>	Super Yoghurt study 043722 3646 <a href="mailto:fatemeh.miremadi@live.vu.edu.au">fatemeh.miremadi@live.vu.edu.au</a>	Super Yoghurt study 043722 3646 <a href="mailto:fatemeh.miremadi@live.vu.edu.au">fatemeh.miremadi@live.vu.edu.au</a>	Super Yoghurt study 043722 3646 <a href="mailto:fatemeh.miremadi@live.vu.edu.au">fatemeh.miremadi@live.vu.edu.au</a>	Super Yoghurt study 043722 3646 <a href="mailto:fatemeh.miremadi@live.vu.edu.au">fatemeh.miremadi@live.vu.edu.au</a>
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# **CONSENT FORM FOR PARTICIPANTS INVOLVED IN RESEARCH TRIAL SCREENING**

## **INFORMATION TO PARTICIPANTS:**

We would like to invite you to undertake preliminary screening as part of a study titled **“The effects of symbiotic yoghurt on lipid profile in mildly to moderately hypercholesterolemic men and women”**, conducted by the College of Health and Biomedicine, Victoria University, St Albans as part of a PhD research project belonging to a registered dietitian and student researcher Fatemeh Miremadi.

The study aims to investigate the effects of daily consumption of symbiotic yoghurt on the lipid profile in people with mild to moderate hypercholesteremia (cholesterol level less than 6.2 mmol/L and triglyceride level less than 2.3 mmol/L), over 8 weeks.

The screening process identifies whether participants meet the requirements of the study's inclusion and exclusion criteria. We are seeking participants that are men and women aged between 30 and 65, and considered overweight or have obesity, by either Body Mass Index (BMI) of between 25 and 35 kg/m<sup>2</sup>, or have a waist circumference of greater than 80 cm, and meet the exclusion criteria specified in the “Information to Participants” form.

During the screening process, participants will be required to complete a comprehensive medical history form, encompassing personal and family medical history, current medication and supplementation usage, allergies, social history, exercise activities and usual food intake. During this consultation, height, weight, waist and hip circumferences, blood pressure and heart rate will also be measured. This consultation will be conducted by

the Student Researcher (a qualified Dietician). The participant also has the opportunity to ask any questions at this time. Following the screening appointment, if participants meet the inclusion and exclusion criteria, consent forms for trial participation may be offered. The trial consent forms may be completed at that time, or taken away for further consideration.

During the study itself, we will be measuring height, weight, waist and hip circumference, blood pressure and taking 3 blood samples over approximately 8 weeks (beginning of study, week 4 and at the end of study, week 8) to measure serum concentration of total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG).

Symbiotic yoghurt is a mixture of a probiotic (beneficial bacteria) and a prebiotic (carbohydrates) that are associated with favourable health effects. Probiotic microorganism has the ability of cholesterol assimilation that may reduce the serum concentration of LDL cholesterol and triglycerides, and increase the beneficial HDL level; however the effects of consuming symbiotic yoghurts compared with regular yoghurts are unclear.

Risks involved in the project are considered minimal, however there are some risks associated which are also detailed in the 'Information to Participants' form.

## **CERTIFICATION BY SUBJECT**

I,  
of

certify that I am at least 18 years old\* and that I am voluntarily giving my consent to participate in screening for the study: **"The effects of symbiotic yoghurt on lipid profile in mildly to moderately hypercholesterolemic men and women"** being conducted at Victoria University overseen by Professor Lily Stojanovska and Associate Professor Michael Mathai.

I certify that the objectives of the study screening, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by the PhD student researcher Fatemeh Miremadi, and that I freely consent to participation involving the below mentioned procedures:

- Complete a comprehensive medical history form, encompassing personal and family medical history, current medication and supplementation usage, allergies, social history, exercise activities and usual food intake.
- Undergo measurements for height, weight, waist and hip circumferences, blood pressure and heart rate

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

I have been informed that the information I provide will be kept confidential.

Signed:

Date:

Any queries about your participation in this project may be directed to the Research Team:

4. Professor Lily Stojanovska, Principal Researcher, College of Health and Biomedicine, Victoria University, Ph: 9919 2737.
5. Associate Professor Michael Mathai, Associate Investigator, College of Health and Biomedicine, Victoria University, Ph: 9919 2211
6. Mrs Fatemeh Miremadi, PhD Research Student, College of Health and Biomedicine, Victoria University, Ph: 0437 22 3646

If you have any queries or complaints about the way you have been treated, you may contact the Research Ethics and Biosafety Manager, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 or phone (03) 9919 4148.

# INFORMATION TO PARTICIPANTS INVOLVED IN RESEARCH

## You are invited to participate

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You are invited to participate in a research project entitled

**“The effects of symbiotic yoghurt on lipid profile in mildly to moderately hypercholesterolemic men and women.”**

This project is being conducted by a student researcher Fatemeh Miremadi as part of a PhD study at Victoria University under the supervision of Professor Lily Stojanovska, and A/Professor Michael Mathai, from the College of Health and Biomedicine.

## Project explanation

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In recent years, interest has risen in the possibility of using the combination of probiotics and prebiotics in the development of new functional foods as they may confer various health benefits including hypocholesterolemic and anti-hypertensive effects. Probiotics have been defined as ‘live microorganisms’ which when ingested in sufficient amounts, beneficially influence the health of the host by improving the composition of intestinal microflora. Prebiotics (such as fructooligosaccharides (FOS) and inulin) are non-digestible carbohydrate substrates in the diet that are the preferred nutrients for probiotics such as *bifidobacteria* and *lactobacilli* and result in their increased number in the large intestine. The combination of both probiotics and prebiotics has been termed as ‘synbiotic’. Therefore, the aims of this study are to investigate whether the daily consumption of symbiotic yoghurt, a yoghurt containing probiotics and prebiotics can improve: 1) serum lipoprotein composition (reduction in LDL-C and TG and increase in HDL-C), and 2) systolic blood pressure (SBP) and diastolic blood pressure (DBP) in hypercholesterolemic men and women (total cholesterol level < 6.2 mmol/L and triglyceride level < 2.3 mmol/L), over 8 weeks.

## What will I be asked to do?

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Participants are invited to take part in an 8 week trial, examining the effect of three different types of yoghurt on lipid profile and blood pressure.

Three types of yoghurt will be trialled and each participant will be randomly assigned into one of these 3 groups to consume daily 300 g of either 1) regular/standard yoghurt, 2) symbiotic yoghurt containing probiotic and prebiotic and 3) flavoured symbiotic yoghurt containing, probiotic, prebiotic and fresh pomegranate juice, over 8 weeks. Both the participants and researchers will be “blinded” as to which yoghurt is being consumed. All three types of yoghurt will be produced in the Victoria University Food Laboratory- Werribee Campus.

Briefly, after a preliminary consultation and consent forms before the trial start, participants will attend the clinic fasted for fasting blood samples and anthropometric measurements [include height, weight, body mass index (BMI), waist and hip circumference, blood pressure and heart rate] at three time points: beginning of the study, week 4 and at the end of the study, week 8. The blood samples will be drawn by intravenous catheter to measure serum concentrations of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), using commercially available kits.



For more information, see “How will this project be conducted” on page 3.

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### **What will I gain from participating?**

There will be no financial gain or personal benefit from participating in this study. By participating in this study, you will be helping researchers understand how consuming symbiotic yoghurts affect the health of hypercholesterolemic people. Studies have shown that there may be health benefits of consuming symbiotic yoghurts, but more research is needed to add to what is currently understood, particularly about the health effects of daily consumption of symbiotic yoghurts. If health benefits are found, then it will provide a practical, economical and convenient recommendation for people with high cholesterol levels and their health care team to consider when developing health care plans.

There will be no payment made to participants, and travel costs to the University will be at the participant's own expense, however a voucher for 2 nutrition consultations at the VU teaching clinic will be issued at the trial completion.

Studies have shown that hypercholesteremia is associated with development of cardiovascular disease (CVD), which is one of the leading causes of death and disabilities in developed countries. Clinical trials have shown that for each 1 mmol higher than normal cholesterol level ( $< 5.5$  mmol/L), the risk of coronary heart disease could increase by approximately 35%, while even a small reduction in serum cholesterol of 1 per cent was found to reduce the risk of coronary heart disease by 2 to 3%. Lactic acid bacteria especially *lactobacilli* are probiotics that have been considered potentially useful in their role in reducing serum cholesterol. Probiotic growth is enhanced by prebiotics that beneficially affect the quality and quantity of those probiotics which are already dominant in the human intestine, and thereby improve host health. Therefore, the selection of potential probiotic strains that would be capable of performing effectively in the gastro-intestinal tract is a significant challenge and needs further investigation.

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### **How will the information I give be used?**

The information gathered by the researcher will be handled with strict confidentiality and statistically assessed at the completion of the study. No names or other identifying details will be made available to anyone but the researchers. Data from the study will be used as part of the researcher's PhD Thesis and will be presented at University presentations and may also be presented at relevant conferences. Scientific publications may also arise out of the data. Furthermore, de-identified data (coded) will be accessible by a statistician, and potential future project students under the supervision of the research team.

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### **What are the potential risks of participating in this project?**

Whilst yoghurt is one of the commonly consumed dairy products, there are some small risks associated with consuming the product. There are also some small risks associated with the study procedures.

- Allergy or intolerance to lactose and dairy products. This will be minimised by screening to ensure participants do not have any allergies to those components.
- Small risks associated with blood taking include: discomfort, bruising, pain, excessive bleeding, fainting or feeling light headed, hematoma (blood accumulating under the skin) and infection. If participant experience any problems, staff will be accessible.
- The anthropometric measurements that the participants will undergo may cause distress, for instance subjects may become anxious about getting their waist circumference measured. Also there is a small risk that the blood sampling may induce procedure related stress and anxiety.

## How will this project be conducted?

<b>BEFORE TRIAL At least 1 week before trial starts</b>		
1.	<b>Considering your involvement</b> <ul style="list-style-type: none"> <li>Review the Information to Participants Form and ask questions about involvement in the trial.</li> <li>Consult with family, friends and medical practitioners if necessary to help make your decision.</li> <li>If you decide to participate, contact the trial research team and make an appointment for pre-trial screening.</li> </ul>	
2.	<b>Pre-trial screening: attend clinic at St Albans Campus</b> <ul style="list-style-type: none"> <li>Fill in medical forms and have anthropometric measures to determine eligibility (height, weight, waist and hip circumference, blood pressure and heart rate).</li> <li>Consult with the researcher and ask any questions.</li> <li>If you are eligible, you may be invited to participate in the trial. If you agree, you will be provided with consent forms that require your signature.</li> <li><b><i>You are not obliged to make any decision at the time and you are free to discuss the decision with friends, family or medical personnel should you wish and advise your decision at a later stage.</i></b></li> </ul>	1.5 hour
3.	<ul style="list-style-type: none"> <li>Sign consent forms to participate, decline the offer or decide later.</li> </ul>	
4.	<ul style="list-style-type: none"> <li>Research team will randomly allocate you to any of the three test group and an appointment made to start the trial.</li> </ul>	
<b>TRIAL STARTS</b>		
At the beginning of Week 1	<ul style="list-style-type: none"> <li>24-hours prior to the trial commencement, avoid vigorous physical activity that is not usually part of your routine</li> <li>Do not consume anything after 10 pm.</li> <li>Arrive at the clinic fasted, only drink warm water.</li> <li>Meet with research team and answer short questions about whether you can take part in the trial that day.</li> <li>Measure height, weight, waist, hip, blood pressure and heart rate.</li> <li>Insert IV cannula into arm</li> <li>Take blood samples</li> </ul>	1.5 hour
Week 4	<ul style="list-style-type: none"> <li>Do not consume anything after 10 pm.</li> <li>Arrive at the clinic fasted, only drink warm water.</li> <li>Meet with research team and answer short questions about whether you can take part in the trial that day.</li> <li>Measure height, weight, waist, hip, blood pressure and heart rate</li> <li>Insert IV cannula into arm</li> <li>Take blood samples</li> </ul>	
At the end of Week 8	<ul style="list-style-type: none"> <li>Do not consume anything after 10 pm.</li> <li>Arrive at the clinic fasted, only drink warm water.</li> <li>Meet with research team and answer short questions about whether you can take part in the trial that day</li> <li>Measure height, weight, waist, hip, blood pressure and heart rate</li> <li>Insert IV cannula into arm</li> <li>Take blood samples</li> </ul>	1.5hour
<b>TRIAL END</b>	Total hours:	6 hours
<b>Please Note: you are free to withdraw from the trial at any time during the trial</b>		

### Who is conducting the study?

---

#### Chief Investigator:-

Professor Lily Stojanovska MSc, PhD  
Victoria University  
Phone +61 3 9919 2737  
Fax 61 +3 9919 2465  
Email. lily.stojanovska@vu.edu.au

#### Associate Investigator:-

Associate Professor Michael Mathai BSc Hons, PhD  
Victoria University  
Phone +61 3 9919 2211  
Fax 61 +3 9919 2465  
Email. michael.mathai@vu.edu.au

#### Student Researcher:-

Fatemeh Miremadi  
BSc(Nutrition Sciences), MNutDiet  
Victoria University  
Phone:9919 8109  
Email :fatemeh.miremadi@live.vu.edu.au

Any queries about your participation in this project may be directed to the Chief Investigator listed above.

If you have any queries or complaints about the way you have been treated, you may contact the Research

Ethics and Biosafety Manager, Victoria University Human Research Ethics Committee, Victoria University

PO Box 1440 Melbourne Vic.

**Medical Background and Dietary Questionnaire:**

First of all, we would like to thank you for participation in this study and ask you to spend some time answering the following questions. Please answer all the questions to the best of your ability as your responses would be extremely valuable for us in order to understand the contributing factors related to hypercholesterolemia and high blood pressure.

This questionnaire is composed of two parts. The first part includes some questions related to your health and medical background. Please answer all the questions to the best of your ability. For the part 1 questions, unless otherwise indicated, select the best choice. Part 2 is your dietary questionnaire which has been previously validated by Cancer Council-Victoria. These questions are about your usual eating habits over the past 12 months. Where possible give only one answer per question for the type of food you eat most often.

As is customary, all of your responses are completely confidential and may only be used in study result summaries and/or reports

**Part 1: Health History Questionnaire**

Name:

Gender:

Age:

DOB:

Address:

E-mail:

1. Have you ever had a definite or suspected heart attack or stroke?  
☐ Yes ☐ No
2. Do you have any other cardiovascular or pulmonary (lung) disease (other than asthma, allergies, or mitral valve prolapse)?  
☐ Yes ☐ No
3. Do you have a history of: diabetes, kidney, liver disease (circle all that apply)  
☐ Yes ☐ No
4. Have you had surgery or been diagnosed with any disease in the past 3 months  
☐ Yes ☐ No  
If yes, please specify the date and type of Surgery/disease.  
Date: \_\_\_\_\_ Surgery/disease: \_\_\_\_\_
5. How long you have had abnormal lipid level (e.g. high cholesterol level)?  
\_\_\_\_\_ month/ \_\_\_\_\_ year
6. Do you currently smoke cigarettes or have quit within the past 6 months?

☐ Yes ☐ No

7. Have your father or brother(s) had heart disease prior to age 55 OR mother or sister(s) had heart disease prior to age 65?

☐ Yes ☐ No

8. Within the past 12 months, has a health professional told you that you have high blood pressure (systolic  $\geq 140$  OR diastolic  $\geq 90$ )?

☐ Yes ☐ No

9. If you have high blood pressure, do you take any medicines to control your blood pressure?

☐ Yes ☐ No

If Yes, please specify :

10. Have you ever been told by a health professional that you have a fasting blood glucose  $\geq 6.0$  mmol/L?

☐ Yes ☐ No

11. Describe your regular physical activity or exercise program:

- Type: \_\_\_\_\_
- Frequency: \_\_\_\_\_ days per week
- Duration: \_\_\_\_\_ minutes
- Intensity (circle one): low moderate high

12. Have you been told by a health professional that you should not exercise?

☐ Yes ☐ No

If Yes, Please specify the reason

13. Are you currently being treated for any other medical condition by a physician?

☐ Yes ☐ No

If yes please provide the details: \_\_\_\_\_

14. Are you currently taking any medication?

☐ Yes ☐ No

If yes please provide the details: \_\_\_\_\_

15. Do any of the following apply to your everyday diet?

- ☐ I eat a lot of pre-prepared/package meals
- ☐ I eat a lot of fast food
- ☐ I eat a lot of sweets

16. Are you currently following a medically prescribed diet? (E.g. diabetes, low salt diet, fluid restricted diet):

☐ Yes ☐ No

If so, which one? \_\_\_\_\_

17. Have you been tested for food intolerances in the past?

☐ Yes ☐ No

If so, which foods? Which test procedure was applied? \_\_\_\_\_

18. Are you currently on any kind of restricted diet?

☐ Gluten Free

☐ Dairy Free

☐ Vegetarian

☐ Vegan

19. Do you know of any foods that cause you to have an allergic reaction?

☐ Yes ☐ No

Please specify any known food allergies: \_\_\_\_\_

**“Thank you for your time, once you have finished this part, please ask the researcher to provide you with your Dietary Questionnaire”**