Antioxidant activity of pomegranate (*Punica granatum* L.) polyphenols and their stability in probiotic yoghurt

A thesis submitted in fulfilment of the requirements for the degree of Master of Applied Science (Food Technology)

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DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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ABBREVIATIONS

a*	Redness (a Hunter colour factor)
AA	Antioxidant activity
ABT-5	Mixed culture of *Lactobacillus acidophilus, Bifidobacterium bifidum* and *Streptococcus salivarius* ssp. *thermophilus*
ABTS	2, 2' -azinobis-(3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AC3.5	Probiotic yoghurt made from RSM and ABT-5 supplemented with 3.5% PJC **after** heat treatment
AC6	Probiotic yoghurt made from RSM and ABT-5 supplemented with 6% PJC **after** heat treatment
AEAC	Ascorbic acid equivalent antioxidant capacity
AJ9	Probiotic yoghurt made from milk and ABT-5 supplemented with 9% IPJ (coded IT) **after** heat treatment
AJ13	Probiotic yoghurt made from milk and ABT-5 supplemented with 13% IPJ (coded IT) **after** heat treatment
AJ17	Probiotic yoghurt made from milk and ABT-5 supplemented with 17% IPJ (coded IT) **after** heat treatment
AJ20	Probiotic yoghurt from milk and ABT-5 supplemented with 20% IPJ (coded IT) **after** heat treatment
SY6	Probiotic yoghurt made from RSM and ABT-5 supplemented with 6% pH-adjusted PJC **after** heat treatment
SY10	Probiotic yoghurt made from RSM and ABT-5 supplemented with 10% pH-adjusted PJC **after** heat treatment
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
b*	Yellowness (a Hunter colour factor)
ºB	ºBrix
BB-12	Single strain culture of *Bifidobacterium bifidum*
BC3.5	Probiotic yoghurt made from RSM and ABT-5 supplemented with 3.5% PJC **before** heat treatment

BJ9	Probiotic yoghurt made from milk and ABT-5 supplemented with 9% IPJ (coded IT) **before** heat treatment
c	Chroma value (a Hunter colour factor)
ºC	Degrees Celsius
CFU	Colony forming units
db	Dry basis
DMPD	N,N-dimethyl-p-phenylenediamine dihydrochloride
DPPH	2,2 diphenyl-1-picrylhydrazyl
DVS	Direct vat set
F-C	Folin-Ciocalteu
FD	Freeze-dried
FDYP	Freeze-dried yoghurt powder
FRAP	Ferric reducing ability of plasma
g	Gram
h	Hours
Hº	Hue angle (a Hunter colour factor)
ha	Hectares
HTs	Hydrolysable tannins
IPJ	Imported pomegranate juice
kg	Kilo grams
kPa	Kilo Pascals
L	Liters
L*	Lightness (a Hunter colour factor)
LA-5	Single strain culture of *Lactobacillus acidophilus*
LHSMP	Low-heat skim milk powder
LPJ	Pomegranate juice from the **large** size '*Wonderful*' variety fruit
M	Molar
mg	Milligrams
min	Minutes
mL	Millilitres
mM	Millimolar
mm	Millimetres
M-Press	Manual press

MRS	de Man-Rogosa-Sharpe
µg	Micrograms
µL	Microlitres
N	Newtons
NaOH	Sodium hydroxide
ORAC	Oxygen radical absorbing capacity
PJ	Pomegranate juice concentrate
PJ1	Juice extracted from manually separated arils with an electrical juicer in two stages
PJ1P	Pasteurized PJ1
PJ2	Juice extracted from manually separated arils by manual pressing
PJ2P	Pasteurized PJ2
PJ3	Juice extracted from peeled, segmented fruit with an electrical juicer in single stages
PJ3P	Pasteurized PJ3
PJ4	Juice extracted from peeled, segmented fruit with an electrical juicer in two stages followed by manual pressing
PJ4P	Pasteurized PJ4
PJ5	Juice extracted from chopped whole fruit with an electrical juicer in single stages
PJ5P	Pasteurized PJ5
PJ6	Juice extracted from chopped whole fruit with an electrical juicer in two stages followed by manual pressing
PJ6P	Pasteurized PJ6
PJC	Pomegranate juice concentrate
PLB	Reconstituted skim milk fermented with BB-12
PLL	Reconstituted skim milk fermented with LA-5
PLM	Control yoghurt made from milk and ABT-5
PLR	Control yoghurt made from RSM and ABT-5
PLS	Reconstituted skim milk fermented with ST-B01
POB	Reconstituted skim milk supplemented with 6% PJC and with BB-12
POL	Reconstituted skim milk supplemented with 6% PJC and with LA-5

POS	Reconstituted skim milk supplemented with 6% PJC and with ST-B01
r	Repeatability limit
R^2	Coefficient of determination for a regression curve
RT	Room temperature
RSM	Reconstituted skim milk
S	Seconds
SPJ	Pomegranate juice from the **small** size '*Wonderful*' variety fruit
SS	Soluble solids
ST-B01	Single strain culture of *Streptococcus salivarius* ssp. *Thermophilus*
t	Tonnes
TA	Titratable acidity
TAC	Total anthocyanins content
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic compounds
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TS	Total solids
UF	Ultrafiltration

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ABSTRACT

Pomegranate is a shrub or small tree grown in different parts of the world that contains higher levels of antioxidants than most fruits. Pomegranate juice (PJ) is well known for its health beneficial compounds, which can be attributed to its total polyphenol compounds and high level of antioxidant activity.

This study was undertaken to characterise the antioxidant properties of the fresh juice extracted from the Australian-grown pomegranate '*Wonderful*' variety in comparison to those reported in the literature and the imported pomegranate juices (IPJs). Total phenolic compounds (TPC) were determined by Folin-Ciocalteu Colorimetric method and expressed as gallic acid equivalent (GAE), while the antioxidant activity (AA) was measured by ABTS method and expressed as Trolox equivalent antioxidant capacity (TEAC). The TPC in the fresh juice was found to be 2,400 ± 200 mg/L GAE with antioxidant activity of 11 ± 1 mM/L TEAC, while the TPC of the four imported juices was in the range of 1,000 - 2,800 mg/L GAE with AA ranging from 5.5 to 14.5 mM/L TEAC.

In an attempt to further improve the TPC levels in fresh juice different parts of the fruit (arils, chopped peeled or whole fruits) were used for juice extraction using six different extraction methods. The extracted juices showed different yield, colour, soluble solids, titratable acidity, TPC and antioxidant levels. Up to five fold increase in TPC and AA level could be achieved by employing intensive extraction on chopped whole fruits. Pasteurisation at 90 ºC for 15 sec did not adversely affect the AA of the resulting samples. Pasteurised juice extracted from arils was

concentrated (PJC) to 52 ^{o}B and added into milk used for the production of a probiotic yoghurt containing selected probiotic bacteria. Reconstituted skim milk (16% TS) was supplemented with 6% PJC and inoculated with mixed culture (ABT-5) containing *Lactobacillus acidophilus* (LA-5), *Bifidobacterium bifidum* (BB-12) and *Streptococcus salivarius* ssp. *thermophilus* (TS-B01). The survival of lactic and probiotic cultures in yoghurts were investigated on weekly intervals during the shelf life of 28 days at 4 ºC. Results obtained indicated more than 90% viability of lactic and probiotic cultures after 28 days storage. To evaluate the effects of PJC on individual culture each strain was inoculated in sterilised RSM (16% TS) and incubated overnight at 37 ºC followed by microbiological analyses. The results revealed that the PJC supplementation adversely affected the population of BB-12, but no significant adverse effect was observed on the number of ST-B01 and LA-5. The effects of PJC supplementation on TPC, colour parameters, texture and sensory attributes of probiotic yoghurts were also analysed. The TPC of the probiotic yoghurt containing 6% PJC was found to be 1590 ± 34 mg/L GAE against a background TPC in plain yoghurt of 1153 ± 32 mg/L GAE (i.e. a net increase of *ca*. 430 mg/L GAE).

Developed probiotic yoghurt (AC6) was subjected to freeze-drying to evaluate the effects of this procedure on the TPC and probitic bacteria viability. This process raised the TPC of the freeze-dried samples 5.2 folds, and increased the counts of ST-B01, LA-5 and BB-12 by 0.81, 0.64 and 0.74 log cycle. Considering the recommended polyphenols intake of *ca.* 1 g/day (Baghurst, 2006) and based on the analytical results obtained, this probiotic product offers a pleasant and effective route to increasing the antioxidant intake in our daily diet.

INTRODUCTION

In the past 2 decades there has been a tremendous increase in demand for functional foods among the consumer due to their potential for providing health benefits. There has been an increased interest in determining dietary sources of antioxidant polyphenols. Thus, red fruit juices such as pomegranate, grape and berry juices have received attention due to their antioxidant activity (AA).

Research on the pomegranate as a medicinal and nutritional food source has grown. Pomegranate juice has recently become more popular in Western diet because of the attribution of health benefits. Pomegranate and its derivates such as juice, peel and seeds are rich source of several high-value compounds with beneficial physiological activities. Its high AA has led to applications in functional food formulation, mainly for heart and prostate health.

While the literature has an abundance of research reports on the biochemical attributes and health benefits of pomegranate there seems be to scarcity of scientific data in Australia on pomegranate grown under Australian climatic conditions. The Rural Industries Research and Development Corporation (RIRDC) has identified pomegranate as a potential new crop for Australia (Eccles, 2009). Therefore, this project was undertaken to overcome the existing knowledge gap, at least partly, by elucidating the physicochemical and phytochemical characteristics of the pomegranate variety '*Wonderful*' which is commonly grown in Australia.

The first stage of this study was therefore to characterize the physicochemical and antioxidant properties of pomegranate juice (PJ) in comparison to those reported in the literature and imported PJs. Pomegranate juice extracted from different parts of the fruit (arils, chopped peeled or whole fruits) were analysed for their chemical properties, total phenoloc compounds (TPC) and AA. The effects of heat treatment and concentration on TPC and AA of juice were also studied and processing conditions optimised for maximum retention of functional properties.

To facilitate the consumers' access to the health effects of pomegranate, a dairy-based functional food containing probiotic bacteria was developed. So, the product development stage of this project provides an example of value-added products containing PJ or its concentrate (PJC) in the formulation of probiotic yoghurt. Physicochemical and functional properties of the developed product and the effects of PJC supplementation during the shelf life have been analysed. Finally, the supplemented probiotic yoghurt was subjected to freeze-drying to evaluate the stability of TPC and viability of probiotic bacteria.

The main aims of this study were therefore:

1) Evaluation of the bioactive components in fresh '*Wonderful*' pomegranate Juice
2) Evaluation of the impact of pomegranate juice polyphenols on the activity and survival of probiotic bacteria in probiotic yoghurt

The specific objectives of this study were:

3) Extraction and characterisation of the pomegranate juice in terms of yield,
4) physicochemical properties and bioactive compounds

5) Evaluation of the effects of heat treatment on physicochemical properties and bioactive compounds of fresh pomegranate juice
6) Development of a probiotic yoghurt containing PJ or its concentrate (PJC)
7) Determining the effects of PJ supplementation on the activity of probiotics during fermentation and product shelf life study
8) Characterisation of probiotic yoghurt in terms of physicochemical, microbiological and organoleptic properties and its bioactive compounds

The current thesis is written in six main chapters. The first chapter reviews the literature and provides a description of pomegranate's historical and botanical background, production trends, health benefits, physicochemical and phytochemical properties, varietal and processing factors affecting the fruit's attributes, development of pomegranate-based new products, and supplementation of milk products with pomegranate juice. Chapter 2 covers the details of materials, chemical reagents, equipments and analytical and micobiological methods used in this study. Chapter 3 includes the results of physicochemical and phytochemical analyses of the fresh and imported pomegranate juices and the effects of raw materials, extraction methods and pasteurisation on fresh juice attributes. Development of probiotic yoghurt supplemented with fresh pomegranate juice or its concentrate, the results of physicochemical and phytochemical analyses of supplemented yoghurt and the effects of freeze-drying on yoghurt cultures and total phenolic compounds are discussed in Chapter 4. The overall conclusions and the future directions of research are highlighted in Chapter 5 followed by references and appendices in the final chapter.

CHAPTER 1

REVIEW OF LITERATURE

1.1 Historical background

The pomegranate *(Punica granatum* L.) is one of the oldest fruits that has not changed much throughout the history (Damania, 2005; Heber, 2006). Numerous historical evidences suggest that this fruit was one of the first 5 crops along with Figs, Dates, Olives and Grapes to be cultivated. Its domestication started 3000 - 4000 BC in the North of Iran and Turkey (Lye, 2008) from where it spread to other regions e.g. Mediterranean countries, India and China, possibly through ancient trade routes. The pomegranate cultivation and usages are deeply embedded in human history and its utilization has been found in many ancient cultures as food as well as a medical remedy (Holland and Bar-Ya'akov, 2008). In the Greek mythology it represents life, regeneration and marriage and in Persian mythology Isfandiyar (legendary Persian hero) eats a pomegranate and becomes invincible. In "The Persian Wars" Herodotus mentions "golden pomegranates" adorning the spears of warriors in the Persian phalanx; in Judaism pomegranate seeds are said to number 613-one for each of the Bible's 613 commandments; in Buddhism it is one of the blessed fruits and represents the essence of favourable influences; in China it is widely represented in ceramic art symbolising fertility, abundance, posterity, numerous and virtuous off springs and a blessed future; in Christianity it is a symbol of resurrection and eternal life; and in Islam the heavenly paradise of the Quran describes four gardens with shade, springs, and fruits including the pomegranate (Langley, 2000).

1.2 Botanical description

Pomegranates (*Punica granatum* L.) have been variously placed in the *Lythraceae* or *Punicacea* family,, depending on the taxonomist and whether they are considering morphological or molecular data (Still, 2006).

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 (Eccles, 2009); its leaves are simple and opposite with entire margins and a deciduous habit. The flowers are bisexual, with radial symmetry.

Figure 1.1 Pomegranate flower and fruit

The fruit is classified as a berry (Still, 2006) consisting 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 (Figure 1.2).

Pomegranates tolerate excessive calcium and thrive on alkaline soils, though they grow well in a variety of soils. Optimal conditions for the pomegranate exist in Mediterranean type, arid climates with high exposure to sunlight, annual total precipitation of 170- 560 mm, mild winters with minimal temperature not lower than- 12 ℃ and hot dry summers without precipitation during the last stages of the fruit development. Under such conditions, the fruit can reach its optimal size, colour, and sugar concentration, without the danger of peel splitting (Levin, 2006).

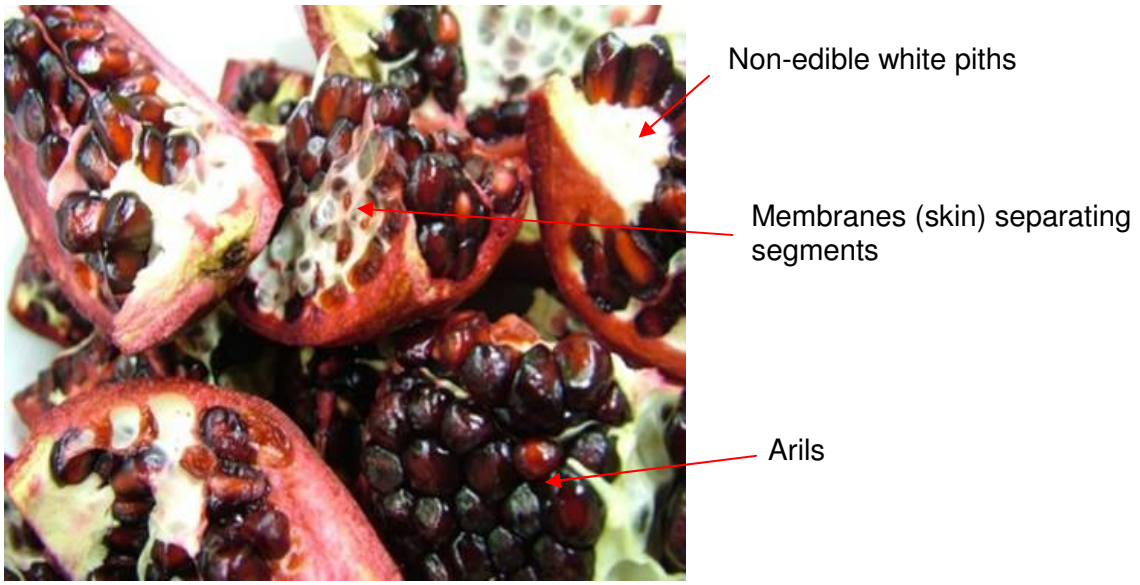


Figure 1.2 Pomegranate fruit's internal structure

A report by the International Plant Genetic Resources Institute (IPGRI, 2001) states that more than 500 pomegranate varieties are known around the world but only 50 are commonly grown. Despite its wide geographic distribution across several continents, very little information is available concerning its genetic origin, centers of diversity, or evaluation of *in situ* and *ex situ* genetic diversity (Table 1.1), and relatively little breeding improvement has been performed (Still, 2006; Verma et al., 2010).

These varieties have a range of quality varying from very sweet to very acidic, with soft to medium-hard or hard seeds. The best quality pomegranates have a good balance of sugar to acid and soft seeds, which can be consumed with the pulp.

Table 1.1 North Hemisphere *ex situ* germplasm collections of pomegranate

Country	Center	Location	Number of Accessions
Albania	Research Institute of Fruit Trees and Vineyard	Tirana	5
China	6 location	Anhui, Henan, Shaanxi, Shandog, Yunnan and Xinjiang	80
EMFTS [a]	11 locations	Italy, Spain	116
France	CIRAD-FLHOR	Capesterre Belle-Eau	2
Germany	Institute of Crop Science Federal Res. Center	Braunschweig	2
	Inst. For Production Nutr. of World Crops	Witzenhausen	ng [b]
Hungary	University of Horticulture & Food Industry	Budapest	3
Iran	Yazd pomegranate collection	Yazd	700
Israel	Newe Ya'ar Research Center	Haifa	60
Portugal	National Fruit Breeding Station	Alcobaca	5
Russia	Vavilov Research Institute of Plant Industry	St. Petersburg	800
Tunisia	Gabes	Tunisia	>60
Turkey	Aegean Agric. Res. Inst.	Izmir	180
	Alata Horticultural Res. Inst.	Mersin	
Turkmenistan	Garrygala Research Station	Garrygala	1117
Ukraine	Nikita Botanical Garden	Yalta	370
U.S.A	National Clonal Germplasm Repository	Davis, CA	200
Uzbekistan	Schroeder Uzbek Research Center	Tashkent	ng [b]

[a] European Minor Fruit Tree Species
[b] Number of accessions not given (Still, 2006; Verma et al., 2010)

A study on pomegranate varieties grown in Australia was conducted at Medina Research Centre of Western Australia in 1990's (Table 1.2).

Table 1.2 Quality parameters of pomegranate varieties grown in Australia

Varieties	Appearance	Internal Quality	Comments
Gulosha Azerbaijani	Large size, good external appearance (light pink/red peel)	Large red grains, juicy	Good variety
Gulosha Rosavaya	Large size, good external appearance (light pink peel)	Large red grains, Juicy	Best variety for combination of sweetness, acidity and external appearance
Wonderful	Medium size, claret red peel	More acidic than Gulosha Rosavay and with red grains	Next best variety to Gulosha varieties, but is smaller and more acidic, most common variety in California, better for juicing
Victorian Giant	Large size, not very attractive peel	Pale grain, not juicy, mild flavour	Poor variety
Berri	Large size, unattractive peel	Not juicy, bland flavour	Poor variety
Veles	Medium size, squarish shape, pink to red peel	Juicy, rich flavour but very acidic	May be suitable for processing
Griffith	Large size Claret red peel	Red grains, rich flavour, but too acidic	Fairly good variety

(Burt & Perth, 2007)

The main variety grown in Australia is the '*Wonderful*' variety (Portman & Johnston, 2008) with large fruits showing deep purple to red peel colour with a glossy appearance and medium thickness. The arils are tender, deep crimson colour with good flavour and soft seeds making the fruit well adapted for both fresh consumption and processing for whole arils or juice (Palou et al., 2007). The consumers prefer blemish-free, medium to large size fruit, with plenty of red colour evident, both externally and in the juicy pulp surrounding a tender seed. Good varieties have a large proportion of edible pulp to rind (non-edible white piths) and cell partition skins, which are unpalatable and bitter (Johnson, 2002).

Most orchards plant pomegranate in low number configurations ranging from 300 to 800 trees per ha with the yield ranging between 10 to 25 t / ha. Some fruit may be seen in year 2 but more commonly in year 3 and the trees are considered mature at about year 7. Fruit is picked 6-7 months after flowering when soluble solids reach 15-17 degree Brix (ºB) depending on the cultivar (Lye, 2008). It is a non-climacteric fruit that does not ripen off the tree even with ethylene treatment, and therefore should be picked when fully ripe to ensure the best flavour (Kader et al., 1984). Pomegranates will store for about 1 to 2 months at ambient temperatures, and can be kept for seven months at 0 to 5 ℃ and 80 to 85 per cent relative humidity (Burt & Perth, 2007).

1.3 Current production trends

1.3.1 Pomegranate world production

As described above, the optimum growth conditions for pomegranate exist in Mediterranean like climates. Thus, the commercial production of pomegranate is

mainly in the Mediterranean basin (such as Turkey, North Africa, Spain and Israel), the Middle-East and Asia (such as, Iran, India and China) and in the New World (the USA, Mexico and Argentina). Although there is no accurate data on world production of pomegranate due to the rapid increase in its production, its current annual production is estimated to be over 1.6 million tons (Table 1.3). Due to ever-increasing consumers' awareness of the direct relationship between food intake and good health - especially from natural foods such as fruits and vegetables - the demand for this fruit is expected to increase in future (Eccles, 2009).

Table 1.3 Estimated world production and trade of pomegranate

Country	Planted area (ha)	Production (t)	Export (t)
Iran	65,000	600,000	60,000
India	54,750	500,000	22,000
China	Unknown	260,000	Unknown
U.S.A.	6,070	110,000	17,000
Turkey	7,600	90,000	Unknown
Spain	2,400	37,000	14,000
Tunisia	2,600	25,000	2,000
Israel	1,500	17,000	4,000
Other [1]	Unknown	Unknown	Unknown

[1] Egypt, Morocco, Chile, Argentina, Australia
(Holland and Bar-Ya'akov, 2008)

1.3.2 Australian market

Pomegranate's use in Australia has been primarily as an ornamental tree (Lye, 2008) and there is no published research about Australian pomegranate or its products. Currently it is estimated that some 250 ha is under pomegranate cultivation expected to double for new plantations. In Australia, pomegranate likes climates with mild to subtropical temperatures (Figure 1.3). It is expected that over the next decade its cultivation will expand to over 1,000 ha with an estimated fruit production value of some $50 M per annum (Eccles, 2009).

Figure 1.3 Pomegranate production areas in Australia (Lye, 2008)

The harvest season is from March to September although in some areas, rain and frost will prevent harvest past late May. New plantation in northern regions could provide Australia with all year-round harvest. There is no organised industry in Australia for pomegranate processing, however, according to Rural Industries Research and Development Corporation (RIRDC), several large commercial developments are taking place (Eccles, 2009). The impediments to its development as a commercial crop have been the lack of access to good varieties

and low consumer demand. This is likely to change with the introduction of new high-yielding, better quality varieties and with increasing market awareness of health foods driving consumer demand (Eccles, 2009).

Considering the rapid world market increase for pomegranate, Australia has a great opportunity to participate in this market as a counter-season producer and exporter. In the last 4-5 years California has shipped over 200 t of pomegranate each year to Australia (Lye, 2008). While there is no current export of Australian pomegranates, there is a four-month window of opportunity for Australian growers-exporters due to counter-seasonal ability to supply a market that is already consuming over 1.6 M t. The Middle-East and Europe represent quite large and ready export markets (Eccles, 2009).

With a wide range of suitable environments, counter seasonality to major producing countries in the Northern Hemisphere and good horticultural expertise in production and marketing, Australia is ideally placed to grow pomegranates. The Rural Industries Research and Development Corporation (RIRDC) have identified pomegranates as a potential new crop for Australia (Eccles, 2009).

1.4 Pomegranate health benefits

Consumers across the world are becoming more interested in foods with health promoting functions as they gain more awareness of the links between food and health (Paseephol and Sherkat, 2009). Epidemiological studies have revealed that consumption of fruits and vegetables with high phenolic content correlates with reduced cardio- and cerebro-vascular diseases and cancer mortality (Hertog et al.,

1997a & b). Phenolic compounds produce their beneficial effects by scavenging free radicals. Recently, there has been an increasing interest in determining dietary sources of antioxidant phenolics, and red fruits and their juices such as grape and different berry juices have received attention due to their high antioxidant activity (Gil et al., 2000).

Pomegranate juice (PJ) has become more popular because of the attribution of important biological actions (Lansky et al., 1998). Numerous studies over the past decade have shown that PJ contains higher levels of antioxidants compared to other fruit juices and beverages (Gil et al., 2000; Tezcan et al., 2009). Seeram et al. (2008) evaluated the commercial polyphenol-rich beverages available in the U.S market for their total phenolic compounds (TPC), antioxidant activity (AA) and antioxidant function on cholesterol (ability to inhibit low-density lipoprotein (LDL) oxidation). The selected beverages included three different brands each of apple juice, black cherry juice, blueberry juice, cranberry juice, Concord grape juice, orange juice, red wines, iced tea beverages, and a pomegranate juice (POM). The ranking of the beverages on the basis of the average amounts of TPC was: PJ > red wine > Concord 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. Among all the beverages tested, PJ showed 20% more antioxidant potency composite index (calculated by assigning each test equal weight).

Because of the extensive knowledge available on pomegranate's health attributes and the increasing public interest in functional foods, the demand for the

pomegranate fruit and its products has tremendously increased in the Western world (Tzulker et al., 2007). Consumption of pomegranates in the form of juice is very popular in various parts of the world, especially in Eastern and Middle-Eastern countries where it is produced in large quantities. Other commercial pomegranate products include jams, jellies, sauces, pastes and wines. Consequently, large number of industries for extraction, processing and packaging of PJ as well as pharmaceutical companies for isolating health promoting compounds from the fruit are developed in these countries (Lansky et al., 1998; Seeram et al., 2006a).

According to Tezcan et al. (2009), clinical studies suggest that PJ increases the activity of serum high-density lipoprotein (HDL)-associated paraoxonase 1 (PON 1) and decreases the low-density lipoprotein's (LDL) susceptibility to aggregation and oxidation (Figure 1.4). According to Rosenblat and Aviram, (2006) PJ can inhibit LDL oxidation in 3 ways:

1. Pomegranate juice polyphenols inhibit copper ion-induced LDL oxidation, and thus reduces the oxidized LDL content.
2. Pomegranate juice polyphenols also increase the activity of serum HDL-associated paraoxonase 1 (PON1).
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.

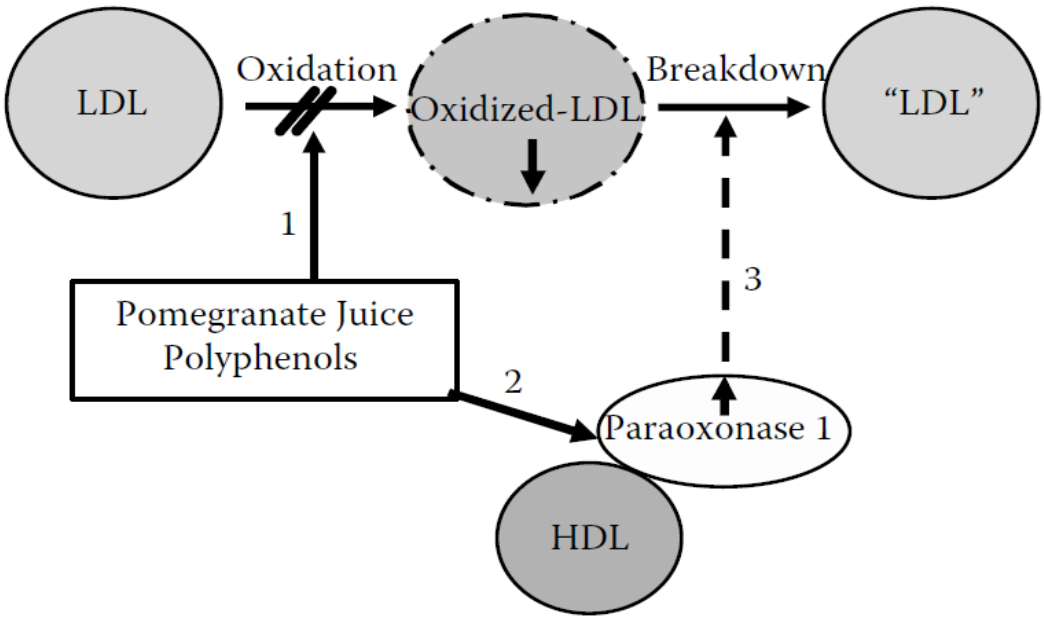


Figure 1.4 Mechanism of LDL oxidation Inhibition by pomegranate juice polyphenols (Rosenblat & Aviram, 2006)

Pomegranate and its products also exhibit strong activity against some species of bacteria such as *Bacillus subtilis, Shigella, Salmonella, Staphylococcus aureus, Vibrio cholera*, *Escherichia coli* and *Yersinia enterocolitica* which justifies its use as a biopreservative in food (Al-Zoreky 2009; Aviram 2002; Aviram and Dornfeld, 2001; Aviram et al., 2000 & 2004; Kaplan et al., 2001; Naz et al., 2007; Plumb et al., 2002; Singh et al., 2002). The PJ consumption also helps keep the prostate specific antigen (PSA) levels stable in men and even slows its rise by extending the PSA doubling time (Pantuck et al., 2006). PJ is also helpful against heart disease (Aviram et al., 2008; Sumner et al., 2005), Alzheimer's disease (Singh et al., 2008), and some types of cancer such as prostate and colon cancers (Adams et al., 2006; Adhami and Mukhtar, 2007; Khan et al., 2007; Malik and Mukhtar, 2006; Seeram et al., 2007).

Turk et al. (2008) reported that PJ consumption improved sperm quality in rats, while Forest et al. (2007) reported improvement in erectile dysfunction in male patients. Pomegranate juice and peel is reported to provide protection against hepatotoxicity (Chidambara Murthy et al., 2002), methicillin-resistant *Staphylococcus aureus* (Machado et al., 2002), and HIV (Lee and Watson, 1998), genital herpes virus (Zhang et al., 1995), and to exhibit estrogen-like activity (Maru et al., 2001) and reduce systolic blood pressure (Aviram and Dornfeld, 2001).

1.5 Antioxidants in plant kingdom

The health benefits attributed to the consumption of fruits, vegetables and cereals are related, at least in part, 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, allium compounds, glucosinolates and indoles, dithiolthiones, isothiocyanates, protease inhibitors, fiber and folic acid. These compounds may act independently or in combination as anti-cancer or cardio-protective agents by a variety of mechanisms. One such protective mechanism is radical-scavenging activity (Rice-Evans et al., 1997). For a compound to be defined as an antioxidant it must satisfy two basic conditions:

1. When present at low concentration relative to the substrate to be oxidized, it can delay or prevent autoxidation or free radical-mediated oxidation.
2. The resulting radical formed after scavenging must be stable in order to interrupt the oxidation chain reaction (Rosenblat and Aviram, 2006).

The autoxidation involves a free-radical chain process that can be initiated by the action of external agents such as heat, light or ionizing radiation (Ingold, 1961), and involves the following steps:

1) *Initiation*

$$RH \xrightarrow{\text{activation}} R^{\bullet} + H^{\bullet}$$

2) *Propagation*

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$$

3) *Chain-breaking termination* (*Inhibition)*

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

Where, RH is the organic substrate; $ROO^{\bullet}$ the corresponding peroxyl radical; ROOH the hydroperoxide; AH the antioxidant; $A^{\bullet}$ the stable, comparatively un-reactive radical

Phytochemicals are the secondary metabolites produced by plants as a defence mechanism against environmental threats such as 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 widespread virtually 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 and Gillespie, 2007).

1.6 Pomegranate polyphenols

Different types of phytochemicals have been identified in various parts of the pomegranate tree, including fruits and seeds. The major class of pomegranate phytochemicals is the polyphenols (phenolic rings bearing multiple hydroxyl groups) that predominate in the fruit (Seeram et al., 2006b).

Rosenblat and Aviram (2006) measured the total phenolic compounds (TPC) levels in ethanolic extracts of pomegranate plant parts and found them to decrease in the order of bark > stem > whole fruit juice > leaves, whereas the antioxidant activity using 10 µmol of total polyphenols/L from each of these plant parts (measured by free radical scavenging capacity assay using 1,1-diphenyl-2-picrylhydrazyl) was in the order of bark > whole fruit juice > stem > leaves. These results suggested that different types and levels of phenolic compounds (e.g. flavonoids and hydrolysable tannins) are present in different parts of pomegranate tree each having a specific antioxidant activity.

According to El-Nemr et al. (1990) edible portion of pomegranate fruit represents on average 52% (w/w) of total fruit, comprising 78% juice and 22% seeds. The fresh juice contains 85.4% water (w/v), 10.6% (w/v) total sugars, 1.4% (w/v) pectin, 0.1% (w/v) acid (expressed as citric acid) and 0.7 mg ascorbic acid, 19.6 mg free amino nitrogen and 50 mg ash per 100 mL. The seeds are rich source of total lipids, protein, crude fiber and ash representing 27.2, 13.2, 35.3 and 2.0% (w/w), respectively. Pomegranate seeds also contain 6.0% (w/w) pectin and 4.7% (w/w) total carbohydrates. The iron, cupper, sodium, magnesium and zinc contents of the juice are lower than those of seeds, except potassium, of which 49.2 ppm is

found in the juice.

In commercial PJ which is obtained by hydrostatic pressing of whole fruits, most phytochemicals including polyphenols from arils, seeds and the peels are extracted along with the juice. The predominant polyphenols in commercial PJ's are flavonoids, condensed tannins and hydrolysable tannins (HTs) (Seeram et al., 2006b & 2005; Van Elswijk et al., 2004; Gil et al., 2000; Hernandez et al., 1999; Tanaka et al., 1986 a & b &1985; Santagati et al., 1984):

1. Flavonoids are mainly found in the peel and arils and include:
    a) flavonols (luteolin, quercetin, and kaempferol)
    b) flavanols
    c) anthocyanins (pelargonidin-3-glucoside, cyanidin-3- glucoside, delphinidin 3-glucoside, pelargonidin 3,5-diglucoside, cyanidin 3,5-diglucoside and delphinidin 3,5-diglucoside)
2. Hydrolysable tannins are found in the peel, membrane and pith and include:
    a) gallotannins that are hydrolised to gallic acid and glucose
    b) ellagitannins (ET) that are hydrolysed to ellagic acid (EA) and glucose
3. Condensed tannins are found in the peel and juice
4. Organic acids such as gallic acid, chlorogenic acid and citric acid are mainly found in the juice

According to Gil et al. (2000), HTs are the predominant polyphenols found in commercial PJ and are responsible for approximately 92% of its antioxidant

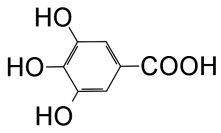
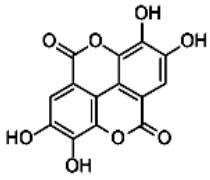
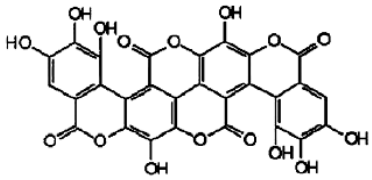
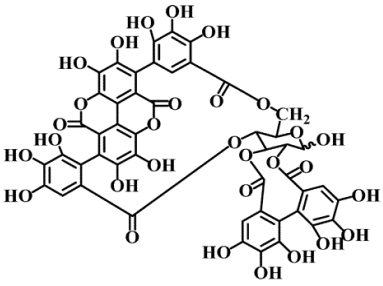
activity. HTs are classified into gallotannins (1,2,4,6-tetra-O-galloyl-D-glucose and 1,2,3,4,6-penta-O-galloyl-D-glucose) and ellagitannins (ellagic acid esters of D-glucose with one or more galloyl substitutions), and are susceptible to enzymatic and non - enzymatic hydrolysis and are further classified according to the products of hydrolysis (Table 1.4). The predominant and the unique gallagyl ester of pomegranate HTs is punicalagin, which is responsible for about half the antioxidant activity of the juice (Seeram et al., 2006a). During industrial juice extraction by hydrostatic pressing the water soluble peel punicalagins are dissolved in the juice, thus contributing to the outstanding antioxidant activity observed in commercial pomegranate juices (Gil et al., 2000).

1.7 Evaluation of total phenolic compounds (TPC) and antioxidant activity in pomegranate juice

Martin et al., (2009) postulated that due to remarkable complexity and heterogeneity of pomegranate polyphenols, the use of gallic acid as a standard leads to significant underestimation of the TPC level. Many available methods of 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.

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

Table 1.4 The building blocks of pomegranate hydrolysable tannins

Molecule	Description of Composition	Chemical Structure
Gallic acid	benzene ring with 1 carboxyl group in position 1 and 3 hydroxyl groups in positions 3, 4 and 5	
Ellagic acid	gallic acid + gallic acid	
Gallagic acid	ellagic acid + ellagic acid	
Punicalagin	gallagic acid + ellagic acid + glucose	

(Martin et al., 2009)

measurement in the F-C assay, where the results are reported as milligram gallic acid equivalents (GAE) (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 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 antioxidant activity (AA) in biological samples. Such methods regard antioxidant activity as an overall characteristic of the product, regardless of the contribution of the individual compound. 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 cause the radicals to disappear (Rice-Evans and Miller, 1994).

In most literatures the AA of PJ is evaluated by five different methods (Cam et al., 2009; Fadavi et al., 2005; Gil et al., 2000; Ozgen et al., 2008; Pande and Akoh, 2009; Tezcan et al., 2009; Tzulker et al., 2007). These methods are:

1- The ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) di-ammonium salt) method,
2- The ORAC (oxygen radical absorbing capacity) method,
3- The DPPH (2,2 diphenyl-1-picrylhydrazyl) method,
4- The DMPD (N,N-dimethyl-p-phenylenediamine dihydrochloride) method and
5- The FRAP (ferric reducing ability of plasma) method

The first four methods are based on the evaluation of the free-radical scavenging capacity of the samples, and the last method is based on measuring sample's iron-reducing capacity (Gil et al., 2000).

The antioxidant activity of the samples are expressed as:

- Inhibition percentage % = [1 – (absorbance $_{Samples}$ /absorbance $_{Control}$)] × 100%
- TEAC (trolox equivalent antioxidant capacity)
- AEAC (ascorbic acid equivalent antioxidant capacity)

TEAC and AEAC represent the concentration (mM) of trolox or ascorbic acid having the antioxidant capacity equivalent to 1.0 mM of the substance under investigation (Antolovich et al., 2002).

Gil et al. (2000) found comparable AA results for PJ samples using DPPH, FRAP and ABTS methods, which were different to those obtained by DMPD method. Proteggente et al. (2002) found a good correlation between the ORAC, FRAP and ABTS methods for various fruits and vegetables (not including pomegranate). Seeram et al. (2008) used DPPH, FRAP, ORAC and ABTS methods to compare the AA of commonly consumed polyphenol-rich beverages in the US market, including PJ. They found similar ranking of the AA of beverages based on the results obtained with DPPH, FRAP and ABTS methods, but ORAC method gave a different rank order.

From the methodological point of view the DPPH and FRAP methods are recommended as easy and accurate methods for measuring the antioxidant activity of fruit and vegetable juices or extracts. The DPPH method is less sensitive than the other methods for hydrophilic antioxidants. The DMPD method should be used with caution in those extracts rich in organic acids. None of the organic acids in PJ showed antioxidant activity when evaluated with the DPPH and FRAP methods. However, citric, malic and tartaric acids showed antioxidant activity with the DMPD method. Citric acid showed a strong neutralising activity on

the DMPD radical, while other organic acids assayed with ABTS, FRAP or DPPH showed considerably less activity (Gil et al., 2000). ORAC method uses a biological relevant free radical (peroxyl radical) and can integrate both degree and time of antioxidant reaction. Technically however, ORAC method requires expensive equipment (fluorometers, which may not be routinely available in analytical laboratories) and long time to quantify the results. This method is also pH and temperature sensitive resulting in significant internal variability. ABTS method on the other hand, is inexpensive, stable to pH and easy to use with fast reaction rate (Priop et al., 2005; Zulueta et al., 2009). The ABTS method can be used for screening of the antioxidant activity of both lipophilic and hydrophilic antioxidants, including, phenolic acids, flavonoids, catechins, L-ascorbic acid, glutathione, uric acid, albumin, bilirubin, cysteine, BHT, α - tocopherol, and plasma antioxidants (Cano et al., 1998; Re et al., 1999; Rice-Evans and Miller, 1995; Rice-Evans et al., 1995 and 1996). For these reasons the ABTS method will be used as a method of choice in this project.

1.7.1 Antioxidant activity determination using ABTS method

The original ABTS assay (TEAC, ferryl myoglobin/ABTS assay) was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation. This has been criticized on the basis that the faster reacting antioxidants might also contribute to the reduction of the ferryl myoglobin radical. Re et al. (1999) modified the ABTS method to a more appropriate form that employed a decolorisation technique. In this method the $ABTS^{+}$ cation is generated directly by oxidation of ABTS with potassium persulfate that is then reduced in the presence of hydrogen-donating antioxidants in a stable form prior to

reaction with putative antioxidants (Figure 1.5).

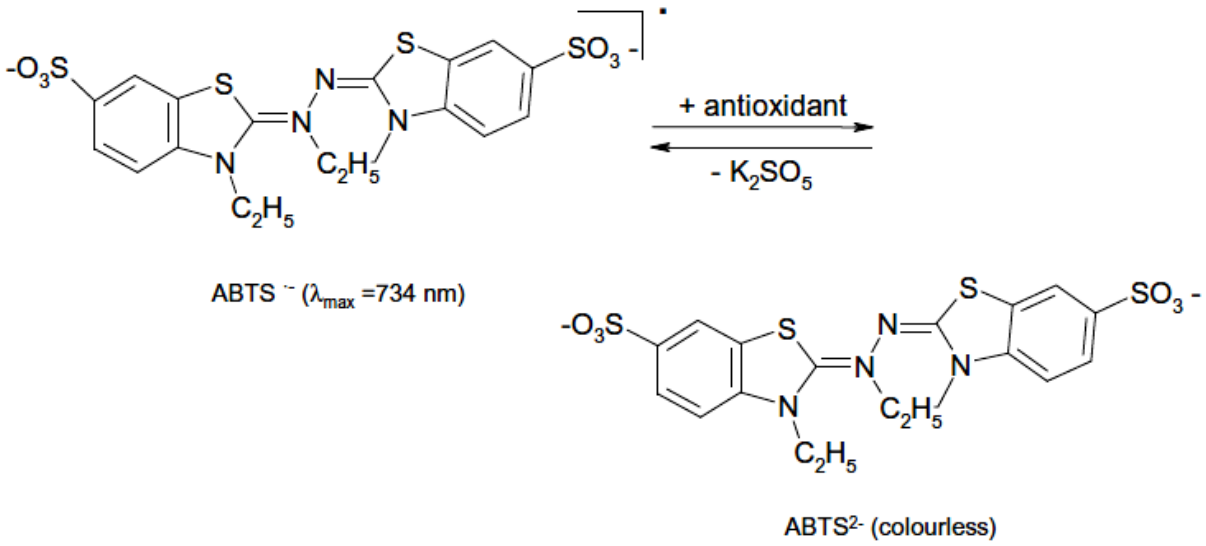


Figure1.5 Reaction of the ABTS radical in the presence of the antioxidant during the ABTS assay (Zulueta et al., 2009)

The ABTS decolorisation technique clearly improves the original TEAC assay (ferryl myoglobin/ABTS assay) for the determination of antioxidant activity in a number of ways. Firstly, the reaction involves the direct generation of the ABTS radical mono-cation with no involvement of an intermediary radical. Secondly, it is a decolorisation assay; thus the radical cation is pre-formed prior to the addition of antioxidant test systems, rather than the concurrent generation of the radical in the presence of the antioxidant. Hence the results obtained with the improved system may not always be directly comparable with those obtained using the original TEAC assay. Thirdly, it is applicable to both aqueous and lipophilic systems (Re et al., 1999).

1.8 Factors affecting pomegranate juice attributes

The level of pomegranate juice attributes i.e. TPC, AA, total anthocyanins content, HTs, soluble solids, acidity, organic acids, sugar, colour, yield and organoleptic properties is influenced by varietal and processing factors.

1.8.1 Effects of varietal and environmental conditions

There is an important body of work in the literature on pomegranate properties from different parts of the world (Table 1.5). These studies have proven that differences in varieties and environmental conditions affect the PJs attributes. The outcomes of these studies are summarised as below.

1.8.1.1 China

Zhuang et al. (2011) analysed the total phenolic compounds (TPC), antioxidant activity (AA), total anthocyanins content (TAC), soluble solid (SS), acidity, sugar and red colour intensity of three Chinese pomegranate cultivars (sour, sweet, red) from Shandong province. They produced pomegranate wine and the correlations among these factors in PJ and wine were determined. Although both PJ and wine showed high level of TPC and AA but some differences existed among these cultivars. The sweet cultivar showed highest TPC and AA while the red cultivar with highest colour intensity had the highest TAC and sugar level. Their results showed a high correlation between AA and TPC and they indicated that phenolic compounds were major contributors to the high antioxidant activity of PJ and pomegranate wine.

Table 1.5 Research publications on pomegranate juice attributes in different countries

Country	Cultivars	Analysed factors	References
Australia	-	-	-
China	3	AA, TPC, TAC, SS, sugars, acidity, colour	Zhuang et al., 2011
Greece	20	AA, TPC, TAC, SS, acidity, colour, juice yield, fruit and seed weight, peel thickness	Drogoudi and Tsipouridis, 2005
Iran	30	AA, TPC, TAC, sugar, protein, mineral, pH, Vit. C, SS, moisture, acidity	Fadavi et al., 2005; Tehranifar et al., 2010
Israel	24	AA, TPC, TAC, SS, HTs, sugars, acidity, colour	Borochov-Neori et al., 2009; Schwartz et al., 2009 a & b
Italy	5	TPC, TAC, SS, pH, acidity	Cristofori et al., 2011
Oman	5	Colour, acidity, Vit. C, juice yield, fruits and arils size, antimicrobial properties	Al-Said et al., 2009; Opara et al., 2009
Spain	40	Sugar, organic acid, organoleptic properties	Melgarejo et al., 2000
Tunisia	30	TAC, organic acids, sugars	Hasnaoui et al., 2011
Turkey	14	AA, TPC, TAC, SS, organic acid, sugars, acidity	Cam et al., 2009; Ozgen et al., 2008
USA	7	AA, TPC, lipid, organic acid, juice yield, fruits and arils volume and weight	Pande and Akoh, 2009; Wetzstein et al., 2011

* AA: antioxidant activity; TPC: total phenolic compounds; TAC: total anthocyanins content, SS: Soluble solids; HTs: hydrolysable tannins

1.8.1.2 Greece

Drogoudi and Tsipouridis (2005) were analysed twenty pomegranate accessions collected from different regions in northern Greece for AA, TPC, TAC, ascorbic acid, SS, acidity, colour and physical properties i.e. fruit and seed weight, peel thickness and juice yield. Their results showed the positive correlation between AA, TPC, TAC, fruit size and ascorbic acid content, while the TAC was negatively correlated with fruit weight. Skin thickness and fruit weight in these samples showed positive correlation with red colour intensity, so they suggested red colour better developed in thick skinned and/or small size pomegranate.

1.8.1.3 Iran

More than 700 accessions of pomegranate have been reported from Yazd pomegranate collection. These have been collected from different part of Iran and obvious similarities in appearance of cultivars were observed among them (Verma et al., 2010). They are mostly late ripening, medium to large size with thick red peel and arils (Zamani, et al., 2007).

Twenty pomegranate cultivars from pomegranate research center (Yazd, Iran) were analysed for AA, TPC, TAC, ascorbic acid, SS, acidity and total sugars by Tehranifar et al. (2010). Their obtained results for analysed parameters were significant different ($P<0.05$) among the investigated cultivars. They indicated that cultivar is the main factor determining the physico and phyto chemical properties in pomegranates.

Juice from ten pomegranate varieties obtained from Saveh Pomegranate

Research Center (Saveh, Iran), were analysed by Fadavi et al. (2005) for their percent of peel, juice and seed and juice chemical attributes including sugars, minerals, vitamin C, protein, pH, acidity and SS of juices. Predominant sugars were fructose (3.50 to 5.96 g/100) and glucose (3.40 to 6.40 g/100). Saccharose and maltose were not practically detected in any variety. The metal ions: K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Pb^{4+} and Cd^{2+} were determined by inductively coupled plasma atomic emission spectrometer (ICP-AES). Contents of K^+, Na^+, Ca^{2+} and Mg^{2+}, were found to be the highest among other fruit juices. The average concentration of vitamin C, protein, acidity, SS and pH of the juice were found to be 0.09–0.40mg/100g, 0.29–1.93 g/100g, 4.0–24.5 g/L, 10.0–16.5 °Brix and 2.90–4.21, respectively (Fadavi et al., 2005).

1.8.1.4 Israel

The changes in the major chemical composition in arils and peels during fruit maturation in two Israeli commercial accessions, '*Wonderful*' and '*Rosh Hapered*' (from Newe Ya'ar research center, Israel) was investigated by Shwartz et al. (2009a). In both accessions, the levels of TPC, AA and hydrolysable tannins (HTs) reduced in the peels during maturation, while the anthocyanin level increased. Their results showed that the sugar content in the aril juice increased in both accessions while the levels of acidity and citric acid decreased during ripening. However, these two accessions differed in other parameters of the aril juice, i.e., while the AA and TPC significantly decreased in '*Rosh-Hapered*', these changes were not observed in '*Wonderful*' accessions. The anthocyanin level, however, increased in '*Wonderful*' but did not change in '*Rosh-Hapered*.' This knowledge could help establish the optimum harvest date ensuring the maximum nutritional

properties of pomegranates.

Schwartz et al. (2009b) described differences in the composition of major ingredients in the arils and peels of 11 accessions grown in Mediterranean and desert climates in Israel. In most of the Mediterranean accessions, the levels of AA and amount of TPC, total anthocyanins content (TAC), SS, glucose, fructose, and acidity were higher in the aril juice compared to those grown in the desert climate. The results indicated that environmental conditions significantly affected pomegranate fruit quality and health promoting compounds.

Fruits of 11 pomegranate cultivars (Experimental farm of the Southern Arava R & D, Israel) were analysed for TPC, AA, SS, acidity and internal red colour intensity by Borochov-Neori et al. (2009). Analyses were carried out at different ripening stages for each cultivar. In three cultivars of different sensory properties and harvest season, comparison between late and early ripening fruit revealed that arils of fruit ripening later in the season contained more TPC and exhibited a higher AA. Multiple linear regression analysis on fruit characteristics in 11 diverse cultivars indicated that juice antioxidative capacity linearly correlated with TPC, but not with the red colour intensity of the arils. Also, no significant correlation was established between aril colour and either juice pH or TPC.

1.8.1.5 Italy

Five pomegranate accessions from experimental farm of Tuscia University (Viterbo, Italy) were analysed for TPC, TAC, SS and acidity by Cristofori et al. (2011). Three of these accessions belong to typology with low-medium acidity and

high sugar content, while the other two belong to typology with high acidity. Based on the fruit size and taste, TPC and TAC level, they recommended the low-medium acidity accessions suitable for fresh consumption in the Italian market, where consumers prefer big fruits and sweet juice while 'high acidity accessions with sour fruits and high TPC level seems particularly suitable for juice production with health properties.

1.8.1.6 Oman

Physical and chemical properties of four pomegranate cultivars in Oman (mountainous area in Northern Oman called Al-Jabal Al-Akhdar) relevant for postharvest handling and processing were determined by Al-Said et al. (2009). Significant differences in fruit size and skin colour, aril size and colour, juice content and acidity were found among the cultivars. On the basis of seeds texture (hardness and toughness), the four pomegranate cultivars studied were classified as 'hard' or 'soft'. Pomegranate cultivars with higher seeds toughness yielded less juice.

Opara et al. (2009) determined the vitamin C content and antimicrobial properties of fresh and dried fractions of peel and arils of one locally grown in Oman (Jabal Al Akhdar) and four imported pomegranates (one Egypt and three Indian varieties). Significant variation in vitamin C content was found among the five varieties studied. Fruit fractions showed antimicrobial effects (inhibition zone) on *Staphylococcus aureus* and *Pseudomonas aeruginosa* but not on *Escherichia coli*. They reported that sun drying of fruit peels significantly enhanced vitamin C retention and antimicrobial effects in comparison with oven drying method.

1.8.1.7 Spain

Melgarejo et al. (2000) analysed the individual organic acids and sugars composition of 40 Spanish pomegranate cultivars (from commercial or experimental orchards, in the Spanish provinces of Alicante and Murcia on Mediterranean coast of Spain) for two consecutive seasons. Three groups of varieties were established according to organoleptic characteristics and chemical composition: sweet, soursweet and sour. Among organic acids citric, malic, oxalic, acetic, fumaric, tartaric and lactic acids were detected, while among sugars glucose, fructose, sucrose and maltose were detected. The average of two seasons' total organic acids and sugars in all 40 cultivars ranged between 0.317 to 2.725 g/100 g (db) and 11.43 to 13.5 g/100 g (db) respectively.

1.8.1.8 Tunisia

Thirty Tunisian pomegranate accessions were analysed by Hasnaoui et al. (2011) for their TAC, organic acids and sugars content. The TAC ranged from 9-115 mg per liter of juice. Among the detected organic acids, malic acid was the major one followed by citric acid, while among sugars, fructose and glucose were the most present in these samples. Two groups of varieties were established based on these results sour and sweet cultivars. They indicated that PJ's sourness or sweetness is due to high or low concentrations of citric acid and not to low or high sugar content.

1.8.1.9 Turkey

The AA, TPC and TAC of PJs obtained from eight cultivars grown in Turkey (Aegean Agricultural Research Institute Experimental Station) were determined by

Cam et al. (2009). Statistically significant differences were observed among some cultivars in terms of AA, TPC and TAC and PJs were classified by cluster analysis into three groups in this regards.

Ozgen et al. (2008) were evaluated the AA, TPC, TAC, SS, acidity, individual sugars and organic acids of six pomegranate cultivars from the Mediterranean region of southern Turkey. The antioxidant capacities averaged between 5.60 to 7.35 mmol TEAC/L; the major sugars found were fructose (6.4 g/100 mL) and glucose (6.8 g/100 mL) and the major acids were citric (1.78 g/100 mL) and malic (0.12 g/100 mL).

1.8.1.10 USA

Six pomegranate cultivars from Ponder farm, (University of Georgia operated farm, Tifton, USA) were investigated by Pande and Akoh (2009) for their AA, TPC, lipid profile and major organic acids in leaves, peel, juice, and seeds. The average AA and TPC were found 26.5 µM TEAC/g and 164.4 mg GAE/100g in fresh PJs. While the peel fraction showed the highest total hydrolysable tannins content in compare to the other fractions, the highest AA and TPC were found in leaves followed by peel, juice and seed, and it showed the positive correlation between AA, TPC.

The compositional changes in pomegranate fruits of the '*Wonderful*' cultivar (Delano, USA), including volume and weight, aril weight and number, pericarp weight, seed weight, and juice/pulp content, were evaluated in fruits of variable sizes by Wetzstein et al. (2011). Their results indicated that because fruit volume

and weight, and total aril weight are closely correlated, any of these characteristics can be used as an indicator of fruit size. The number of arils per fruit was highly correlated with fruit size with larger fruit containing greater numbers of arils. This is in contrast to individual average aril weight, which showed no significant relationship to the fruit size.

1.8.2 Effects of processing on pomegranate attributes

The industrial processing of PJ was developed over the last two decades (Seeram et al., 2006a). Consequently, optimizing the processing technologies and evaluation of their effects on the final products attributes have become a potential research area. These studies have focused on three main areas: (1) raw materials, (2) processing, and (3) storage.

1.8.2.1 Effects of fruit part on PJ attributes

The antioxidant activity of commercial and fresh pomegranate juices was evaluated by Gil et al. (2000). Higher antioxidant activity was found in commercial juices extracted from whole fruits than in experimental juices that were obtained only from the arils. High-performance liquid chromatography coupled with a photodiode array detector (HPLC-DAD) and electro-spray mass spectrometric analyses of these juices revealed that commercial juices contained 1500-1900 mg/L punicalagin (tannin) while only traces of this compound were detected in the fresh juice extracted from the arils. This proved that industrial processing extracted more hydrolysable tannins from the fruit peels resulting in higher antioxidant activity in commercial juices. The relationships between antioxidant tactivity, total phenolic compounds, total anthocyanins content and the levels of four major

hydrolysable tannins in four different juices and homogenates prepared from different parts of pomegranate fruit were studied by Tzulker et al. (2007). In this study 29 different accessions from a large collection in Newe Ya'ar (Northern Israel) were tested. Their results indicated that antioxidant activity in aril juice was significantly correlated to the TPC level and total anthocyanins content (TAC). However, the homogenates prepared from the whole fruit exhibited higher antioxidant activity than the aril juice. Unlike the arils, the antioxidant activity in the homogenates correlated significantly with the content of the four hydrolysable tannins in which punicalagin was predominant, while no correlation was found to the level of anthocyanins.

1.8.2.2 Effects of processing on PJ attributes

The influences of processing and pasteurisation on colour values and TPC content of PJs extracted from pomegranates grown in Izmir (Turkey) were investigated by Alper et al. (2005). Pomegranate juices were clarified by conventional fining alone (flocculated with gelatin and bentonite) or in combination with polyvinylpolypyrrolidone (PVPP) or ultrafiltration (UF) methods, then packed in bottles and pasteurised for 20 min in boiling water. The results showed that clarification methods and heat treatments significantly affected the colour values of PJs, and the TPC reduction was 2.3% for conventional fining, 1.2% for conventional fining together with PVPP, and 14.0% for UF method.

Pomegranate juice produced from Gaziantep cultivars (Turkey) was concentrated using various techniques by Maskan (2006). The final concentration level of 60.5 °Brix was achieved within 23, 108 and 190 min by using either microwave (350 W

power level), rotary vacuum (66 rpm and 40 ℃) and atmospheric evaporation processes, respectively. They investigated colour change during concentration using Hunter L*, a* and b* parameters and found that the severity of pigment destruction was higher in rotary vacuum process compared to other concentration methods used.

1.8.2.3 Effects of storage on PJ attributes

Alighourchi and Barzegar (2009) investigated the variations in TAC, chromatic parameters, pH, titratable acidity, and soluble solids content of reconstituted PJs (13.7 °Brix plus 3% added sugar) made from industrial concentrate followed by pasteurization (93 °C for 30s) and storage for 210 days at 4, 20 and 37 °C. It was shown that while the TAC correlated with some chromatic parameters of the PJs and decreased by increasing storage temperature and time, the soluble solid content and pH increased during storage at all temperatures. They suggested 4 °C as the best temperature for long-term storage which maintained marketing and nutritional qualities of the juices for a reasonable length of time.

1.9 Pomegranate products research and development (R & D)

The commercial potential and economic impact of pomegranates is enormous considering the different ways in which the fruit may be utilised. There is even a greater potential for the use of pomegranate ingredients in functional foods, cosmeceuticals, nutraceuticals, phytoceuticals, and dietary supplements (Seeram et al., 2006a).

The R & D projects using the pomegranate as a functional ingredient can be classified as traditional and new products containing pomegranate (Table 1.6).

Table 1.6 Selected R & D projects using functionality of pomegranate

Project	Analysed factors	References
Traditional products		
Anardana	TAC, techniques improvement	Jaiswal et al., 2010; Kingsly and Singh, 2007; Singh et al., 2007
Traditional PJ concentrate	AA, TPC, sugars, minerals	Orak, 2009
New products		
Blend of PJ and lemon juice	AA, flavonoids, Vit. C, colour	Gonzalez-Molina et al., 2009
Encapsulated PJ	TPC, TAC, encapsulating efficiency	Robert et al., 2010
Fermented PJ	microbial population, pH, acidity, sugar, organic acid	Mousavi et al., 2011;
	AA, TPC, EEIP	Schubert et al., 1999
Cold pressed pomegranate seed oil	AA, TPC, EEIP, fatty acid	Schubert et al., 1999

* AA: antioxidant activity; TPC: total phenolic compounds; TAC: total anthocyanins content; PJ: pomegranate juice; EEIP: eicosanoid enzyme inhibition properties.

1.9.1 Traditional pomegranate products

Pomegranate is one of the oldest edible fruits that have been used not only as fresh fruit and juice but also as processed traditional additives. Recently some of these old products such as “Anardana” and traditional pomegranate juice concentrate have been studied for their functional attributes. The outcomes of these studies are summerised in Appendix 1.

1.9.2 Development of pomegranate-based new products

Considering the huge economic impact of pomegranates on the food and beverage industries (Seeram et al., 2006a), new products containing pomegranate have been developed. Some of these new products such as fermented PJ and mixed fruit juice are consumed directly while others such as encapsulated PJ and seed oil are used as additives in food industry. The outcomes of these studies are summerised in Appendix 2.

1.10 Novel functional foods containing pomegranate

Consumers have become more aware of the relationship between good health and food intake, particularly from naturally derived foods such as fruits, vegetables and probiotic dairy products. These foods are often referred to as functional foods (Eccles, 2009). The concept of ‘‘functional food’’ involves and requires the use of bioactive ingredients or the presence of natural healthy bioactive molecules in foods (Coisson et al., 2005). The European Consensus Document (Bellisle et al., 1998) used the following definition: ‘‘A food can be regarded as ‘‘functional’’ if it satisfactorily demonstrates to affect beneficially 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 and/or reduction of risk of disease“. Pomegranate and its products containing health promoting bioactive compounds could be considered as “functional” ingredients for their anti free radical activities. The PJ or PJC can also be considered as good supplements for foods and dietetics (Tomas-Barberan et al., 2006). According to Paseephol and Sherkat, (2009) among the functional foods, products containing probiotics are showing increasing trends worldwide.

1.10.1 A brief review of probiotics

The word ‘probiotic’, derived from the Greek language, means ‘for life’ (Fuller, 1989) and has had many definitions such as “substances produced by protozoa that stimulate the growth of another” or “organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance” (Lourens-Hattingh and Viljoen, 2001). These general definitions were unsatisfactory because ‘substances’ could include chemicals such as antibiotics. The definition of probiotics has since been expanded to stress the importance of live cells as an essential component of an effective probiotic. Fuller, (1992) defined probiotic foods as “food containing live microorganisms believed to actively enhance health by improving the balance of microflora in the gut”. Kailasapathy and Chin, (2000) believed that there was no one agreed set of selection criteria for classifying a viable bacterial strain as a probiotic but the common criteria used for isolating and defining probiotic bacteria and specific strains has been reported by them as:

(1) genera of human origin; (2) stability against bile, acid, enzyme and oxygen; (3) ability to adhere to intestinal mucosa; (4) colonization potential in the human gastrointestinal tract; (5) production of antimicrobial substances; and (6) demonstrable efficacy and safety.

1.10.2 Yoghurt as a probiotic product

Since the renewed interest in probiotics, different types of products were proposed as carrier foods for probiotics by which consumers can ingest large numbers of their cells for the therapeutic effect. More than 90 probiotic products containing one or more groups of probiotic organisms are available worldwide. A number of

probiotic organisms including *L. acidophilus*, *Bifidobacterium* spp., *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Propionibacteria* are incorporated in dairy foods. These organisms grow slowly in milk during product manufacture (Tharmaraj and Shah, 2003).

Yoghurt is a typical fermented milk consumed all around the world. As a major dairy product it has long been recognised as having desirable health effects, and it is not surprising that most consumers consider yoghurt to be 'healthy' (Lourens-Hattingh and Viljoen, 2001). In Australia, yoghurt consumption has increased steadily from 5.6 kg per capita in 2001 to 7.1 kg in 2010 (Dairy Australia, 2010). This ''biotechnological'' food is considered as having high nutritional value (namely low lactose and high calcium levels) and positive bioactive effects (containing prebiotic ingredients and probiotic bacteria). The ''natural'' plain yoghurt is produced by adding lactic acid bacteria that induce the lactic fermentation (Coisson et al., 2005) and according to Codex Standard (243-2003) yoghurt is classified as fermented milks and could contain a maximum of 50% (m/m) of non-dairy ingredients (such as fruits and vegetables as well as juices, purees, pulps) and its commonly used starter cultures are *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. These cultures do not survive the gastric passage nor colonise the gut but provide their therapeutic benefits via the production 'bacteriocins'. Lactic acid bacteria grow rapidly and thus are added to fermented dairy products as starter cultures to speed up the fermentation process (Shah and Jelen, 1990).

A number of health benefits have been claimed for probiotic bacteria and

consequently, they are increasingly incorporated into dairy foods (Shah 2000). In recent years some yoghurt products (bio-yoghurt) have been reformulated to include live strains of *L. acidophilus* and species of *Bifidobacterium* (known as AB-cultures) (Lourens-Hattingh and Viljoen, 2001). Due to slow growth of AB-cultures normal yoghurt cultures (i.e., *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) are also added to speed up the fermentation rate. The beneficial effects of probiotic bacteria can be expected only when viable cells are ingested (Dave and Shah, 1996). According to Codex Standard (243-2003) to provide health benefits, the concentration of probiotic bacteria should be no les than 10^6 CFU/g of the product.

1.11 Supplementation of milk or milk products with polyphenols

Addition of antioxidants is an emerging trend for 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 (Gad and Abd El-salam, 2010). Polyphenols in most fruits (such as pomegranate) are recognized as the major class of phytochemicals with antioxidant activity (Seeram et al., 2006b). The bioavailability of polyphenols in milk is somewhat controversial (Gad and Abd El-salam, 2010). Some early studies claimed that maximum antioxidant capacity and hence better health benefit could be gained by ingesting milk proteins-phenols complex (Hoffman et al. 2001; Leenen et al. 2000), however, later studies reported reduced bioavailability of phenolics after ingestion with milk (Lorenz et al. 2007; Serafini et al. 2009).

1.11.1 Milk background polyphenols and antioxidant activity

Oxidative reactions in milk are affected by a complex interplay of pro- and antioxidants since many antioxidants can be found in milk and several reactions are possible (Lindmark-Mansson and Akesson, 2000). Among antioxidant enzymes, superoxide dismutase and catalase have been demonstrated in milk (Hoolbrook and Hicks, 1978; Ito and Akuzawa, 1983). Non-enzymatic antioxidants can be formed in the animal body or need to be supplied in the feed as essential nutrients (Focant et al., 1998; St-Laurent et al., 1990). The iron-binding protein lactoferrin can act as an antioxidant, and vitamins C and E (tocopherols and tocotrienols) have antioxidant activity. Some carotenoids have provitamin A action as well as antioxidant functions. Several non-enzymatic antioxidants act as radical scavengers in the lipid phase, such as vitamin E, carotenoids and ubiquinol, whereas vitamin C acts in the water phase. Others can react in both the lipid and the water phase, such as some flavonoids, which operate both as radical scavengers and metal ion binders (Lindmark-Mansson and Akesson, 2000).

Sonmez et al. (2010) determined the AA and TPC content of 6 different brands of UHT milk (plain and flavoured with chocolate or strawberry) using ABTS and F-C methods and found higher AA and TPC in flavoured milk samples. They reported a background AA of 4.31 ± 0.51 mM/mL TEAC and TPC of 1030.10 ± 19.30 mg/L GAE in plain UHT milk. According to O'Connell and Fox, (2001) the background polyphenols may be a consequence of several factors, namely, the consumption of particular fodder by cattle, the catabolism of proteins by bacteria, contamination with sanitising agents, process-induced incorporation or deliberate addition as specific flavouring or functional ingredients. The consumption of polyphenol-rich foods by cattle can affect ruminant health (reduces the incidence of pasture bloat

in the reticulorum - Haslam, 1998) and the yield and quality of milk (fat content and non-casein nitrogen content - Blauwiekel et al., 1997). The specific polyphenols profile of milks from different ruminant species appear to play a significant role in the distinct sensory traits of these milks and their products. The ability of polyphenols to enhance functional properties of milk and dairy products (i.e., microbiological stability, oxidative stability and heat stability) has also been established (Bokukhava et al., 1975; O'Connell et al., 1999 a & b; Rosenthal et al., 1997).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Pomegranate fruit and juice

The fresh '*Wonderful*' pomegranates were obtained from a grower in Robinvale (between Mildura and Swan Hill in the North West of Victoria, Australia, 34° 35' S latitude and 142° 46' E longitude) during the harvest season in April, 2010 and were graded in two groups on the basis of their average weights i.e. 238 ± 10 g and 573 ± 21 g (Figure 2.1).

Figure 2.1 Size grades of the fresh '*Wonderful*' pomegranates

Four brands of imported pomegranate juices (IPJ) were purchased from the local market and coded as TT, TB, UP, IT. Samples TT and IT were sold in 1,000 mL tetra pack cartons, the TB was sold in 1,000 mL bottles and the UP was purchased in 236 mL PET (polyethylene terephthalate) bottles (although other

sizes up to 1 L were also available on the market). Nutritional panels on all purchased products claimed 100% juice with no added ingredients.

2.1.2 Milk and milk powder

For preliminary studies yoghurt batches were prepared from commercial homogenised and pasteurised low-fat milk (1.3% fat, REV, Parmalat, Melbourne, Vic, Australia) that was standardised to 16% solids content with low-heat skim milk powder (LHSMP, 0.9% fat, 36.1% protein, 96% total solids, Bonlac Foods Ltd, Melbourne, Vic, Australia). For subsequent studies LHSMP was reconstituted with Milli-Q water to 16% solids content to produce reconstitute skim milk (RSM).

2.1.3 Starter cultures

The freeze-dried (FD) probiotic cultures selected for this project were supplied by Chr. Hansen Pty. Ltd., (Melbourne, Vic. Australia) and included ABT-5-Probio-Tec™ (a mixture of *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Streptococcus salivarius ssp. thermophilus*), LA-5®- Probio-Tec™ (single strain culture of *Lactobacillus acidophilus*), BB-12 ®-Probio-Tec™ (single strain culture of *Bifidobacterium bifidum*) and ST-B01 (single strain culture of *Streptococcus salivarius ssp.* thermophilus). The cultures were kept at -22 ℃ until required for yoghurt preparation or in modelling studies as direct vat set (DVS) method.

2.1.4 Chemicals, reagents and media

The list of all chemicals, reagent and media used and the respective suppliers are presented in Table 2.1.

Table 2.1 Chemicals, reagent and media used and their suppliers

Chemicals, reagents and media	Suppliers
D-sorbitol	Ajax Chemicals International Pty. Ltd., Sydney, NSW, Australia
Lithium chloride	May & Baker Ltd., Dagenham, UK
de Man, Rogosa & Sharpe Agar (MRS agar), Gas-pack system (AnaeroGen), lactose bacteriological, M17 agar and peptone water medium	Oxoid Australia Pty. Ltd., Adelaide, SA, Australia
ABTS (2, 2' -azinobis-(3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt), cystein hydrochloride, Folin-Ciocalteu reagent (F-C), gallic acid, nalidixic acid, neomycin sulphate, paromomycin sulphate, potassium persulfate (di-potassium peroxdisulphate) and trolox ® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)	Sigma-Aldrich Co, St Louis, MO, USA

2.2 Methods

2.2.1 Samples preparation

2.2.1.1 Extraction of pomegranate juice

Arils of large and small pomegranate fruits were manually separated from the peels and piths and their juice was extracted using an electric juicer (Sunbeam, model IE-AD, Milan, Italy) in two stages (the pulp from first extraction stage was passed through the juicer a second time for further juice extraction). Fresh juices thus extracted from the large and small fruits were pooled separately and labelled as LPJ and SPJ respectively, and stored in a blast freezer at -28 °C for

subsequent physicochemical and biochemical analyses.

To improve juice yield and increase their phytochemical content six different extraction methods were designed and used on small size pomegranates and the resulting juices were coded PJ1 to PJ6 (Figure 2.2). PJ1 was extracted from manually separated arils either with an electric juicer (PJ1) in two stages (as described above) while PJ2 was extracted by pressing the separated arils in a manual screw press (M-Press) (Figure 2.2). The next two batches were extracted after the outer leathery skin of pomegranates were peeled off, the fruit was segmented in four and passed through the electric juicer either in single stage (PJ3), or in two stages followed by manual pressing of the residual pulp (PJ4). For the last two batches, the whole (unpeeled) fruits were chopped and processed in electric juicer either in single stage (PJ5) or in two stages followed by manual pressing (PJ6).The fresh juices thus extracted were pooled separately and after yield calculation each stream was divided into two lots, the first lot (coded PJ1 - PJ6) was frozen and stored in at - 28 ℃ while the second lot (coded PJ1P - PJ6P) was pasteurised as follows before storage at refrigerated temperature.

Figure2.2 Manual screw press

2.2.1.2 Pasteurisation of pomegranate juices

Different streams of fresh juices (PJ1 to PJ6) were bulk pasteurised at 90 ºC for 15 sec, cooled in an ice bath and aseptically transferred into sanitized 450 mL glass bottles, tightly sealed (coded PJ1P to PJ6P) and stored at 4 ºC (Figure 2.2).

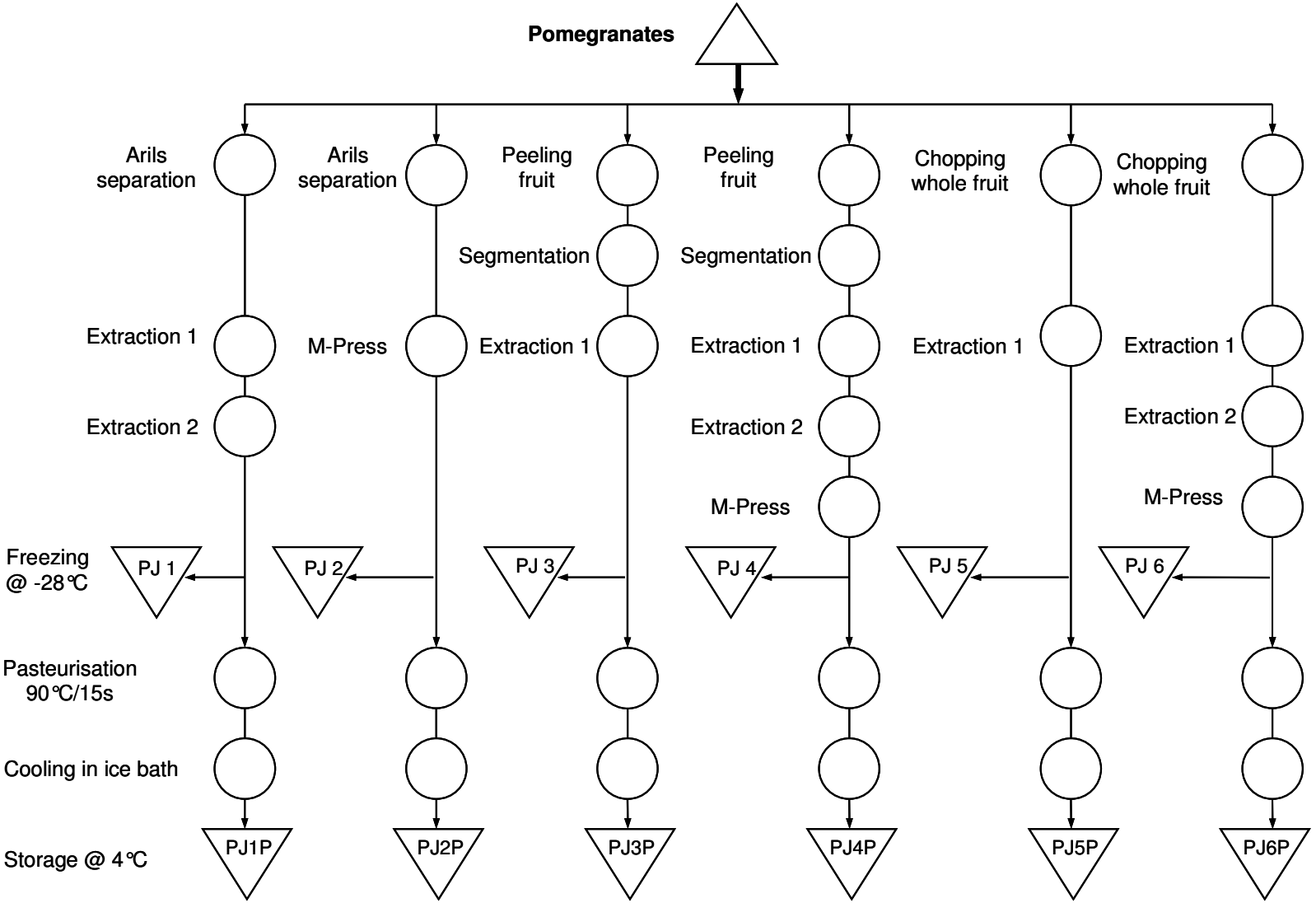


Figure 2.3 Process flow diagram for extraction and processing of juice from different parts of pomegranate fruit

2.2.1.3 Concentration of pomegranate juice

Pomegranate juice coded PJ1P was subsequently concentrated to 52 ^{o}B (coded PJC) using a Climbing Film Evaporator (Jobling, James A Jobling and Co Ltd, Stoke-on-Trent, UK) (Figure 2.3). 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.

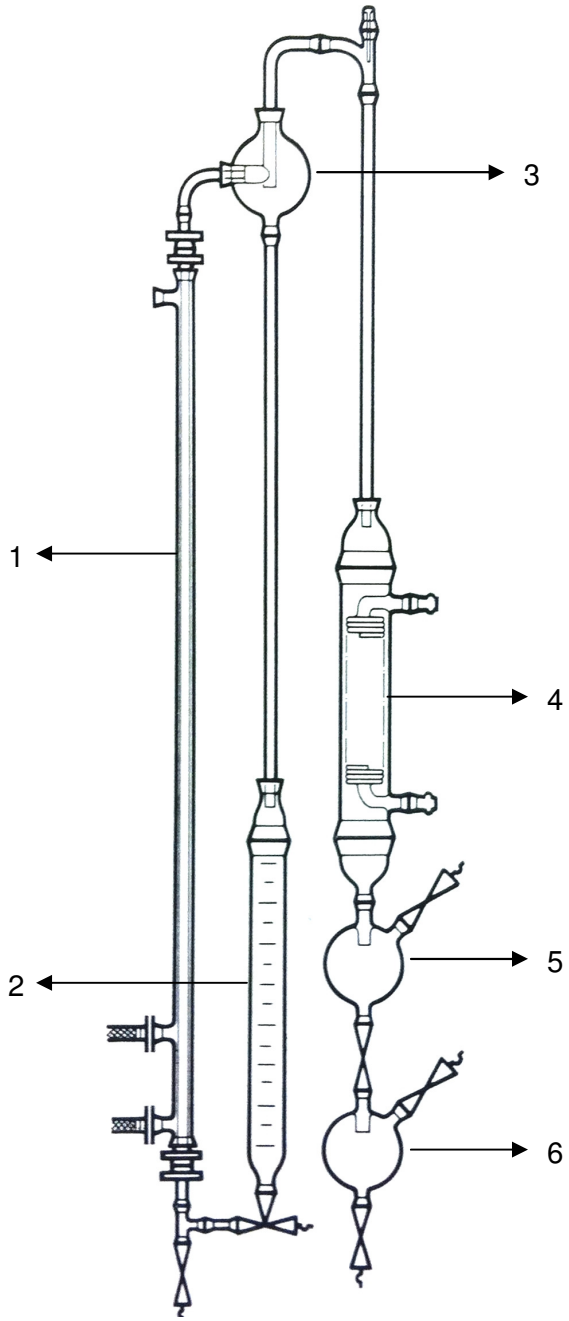


Figure 2.4 Climbing Film Evaporator
1. Calandria, 2. Concentrate receiver, 3. Separator, 4. Condenser, 5 & 6. Condensate receiving vessels

2.2.1.4 Yoghurt production

Yoghurt production protocol is presented in table 2.2.

2.2.1.4.1 Plain yoghurt

The control yoghurt was prepared according to Kusuma et al. (2009). Briefly, the RSM and commercial pasteurised low-fat milk were standardized to 16% solids content using LHSMP, then heat-treated at 90 ℃ for 10 min, cooled to 43 ℃ and aseptically inoculated with freeze-dried ABT-5-Probio-TecTM culture using DVS method at a rate recommended by the supplier (50U/250L). After gentle stirring to distribute the culture evenly, the inoculated milk samples were aseptically transferred into 100-mL plastic containers, tightly sealed and incubated at 43 ℃. Upon reaching pH 4.7 the samples were transferred to a cold room at 4 ℃. Plain yoghurts were coded PLM (from milk) or PLR (from RSM) and used as control samples (Table 2.2).

2.2.1.4.2 Yoghurt supplemented with imported pomegranate juice (IPJ)

Due to seasonal unavailability of local pomegranates when the project started, the commercial imported pomegranate juice (IPJ) coded IT was selected for preliminary supplementation trials to produce probiotic yoghurt with commercial pasteurised low-fat milk. Four supplementation levels of 9, 13, 17 and 20% were selected while keeping solids content constant at 16% by varying the amount of LHSMP added using the following equation:

Solid content = (%LHSMP × 0.96) + (%Milk solids) + (%$PJ_{(IT)}$ × 0.133)

The selected levels of the juice were added either before (coded BJ9) or after

(coded AJ9, AJ13, AJ17 and AJ20) heat treatment (Figure 2.4.a).

2.2.1.4.3 Yoghurt supplemented with concentrated juice (PJC)

The PJC (52 ^{o}B) samples (section 2.2.1.3) were added to yoghurt milk at 3.5 and 6% levels either before (coded BC3.5) or after heat treatment (coded AC3.5 and AC6) (Figure 2.4b). To further increase the supplementation level the PJC pH was adjusted with NaOH (7N) to a final pH of 5 and added at 6 and 10% levels to RSM post heat treatment and incubated as above to produce yoghurt samples coded SY6 and SY10 (Figure 2.4.b). The protein content in these samples was kept constant by adjusting the amount of LHSMP (16%) added in their formulations (Table 2.2).

2.2.1.4.4 Model systems to study the activity of the single strain LA-5, BB-12 and ST-B01 in presence of PJC

RSMs (16% solids content) were sterilised (121 °C for 15 min) and aseptically supplemented with 6% PJC (52 ^{o}B) and inoculated with freeze-dried single strains of LA-5 (coded POL), or BB-12 (POB) or ST-B01 (POS) at a level recommended by the supplier (50U/250L for probiotic bacteria and 50U/500L for ST-B01). After gentle stirring to distribute the culture evenly, the inoculated samples were incubated at 37 °C for 12 h and stored at 4 °C (12 h) followed by microbiological analyses. Non juice-supplemented samples were also prepared with the same strains and used as controls (Table 2.2)

Table 2.2 Yoghurt production protocol

Code	Base	Supplement	Ingredients (%)				Culture type
			LHSMP [1]	Milk	Milli-Q Water	Supplement	
POS	RSM	PJC [2]	16	-	78	6	ST-B01
POL	RSM	PJC	16	-	78	6	LA-5
POB	RSM	PJC	16	-	78	6	BB-12
PLS	RSM	-	16	-	84	0	ST-B01
PLL	RSM	-	16	-	84	0	LA-5
PLB	RSM	-	16	-	84	0	BB-12
BC3.5	RSM	PJC	16	-	80.5	3.5	ABT-5
AC3.5	RSM	PJC	16	-	80.5	3.5	ABT-5
AC6	RSM	PJC	16	-	78	6	ABT-5
SY6	RSM	pH-PJC [3]	16	-	78	6	ABT-5
SY10	RSM	pH-PJC [3]	16	-	74	10	ABT-5
PLR	RSM	-	16	-	84	0	ABT-5
Preliminary studies with milk and IPJ (IT)							
BJ9	Milk	IPJ [4]	7.1	83.9	0	9	ABT-5
AJ9	Milk	IPJ	7.1	83.9	0	9	ABT-5
AJ13	Milk	IPJ	6.9	80.1	0	13	ABT-5
AJ17	Milk	IPJ	6.8	76.2	0	17	ABT-5
AJ20	Milk	IPJ	6.7	73.2	0	20	ABT-5
PLM	Milk	-	7.5	92.5	0	0	ABT-5

[1] LHSMP: Low heat skim milk powder (TS: 96%, Pr: 36.1%), [2] PJC: 52 °Brix, [3] pH-adjusted PJC, [4] IPJ (IT): 13.3 °Brix

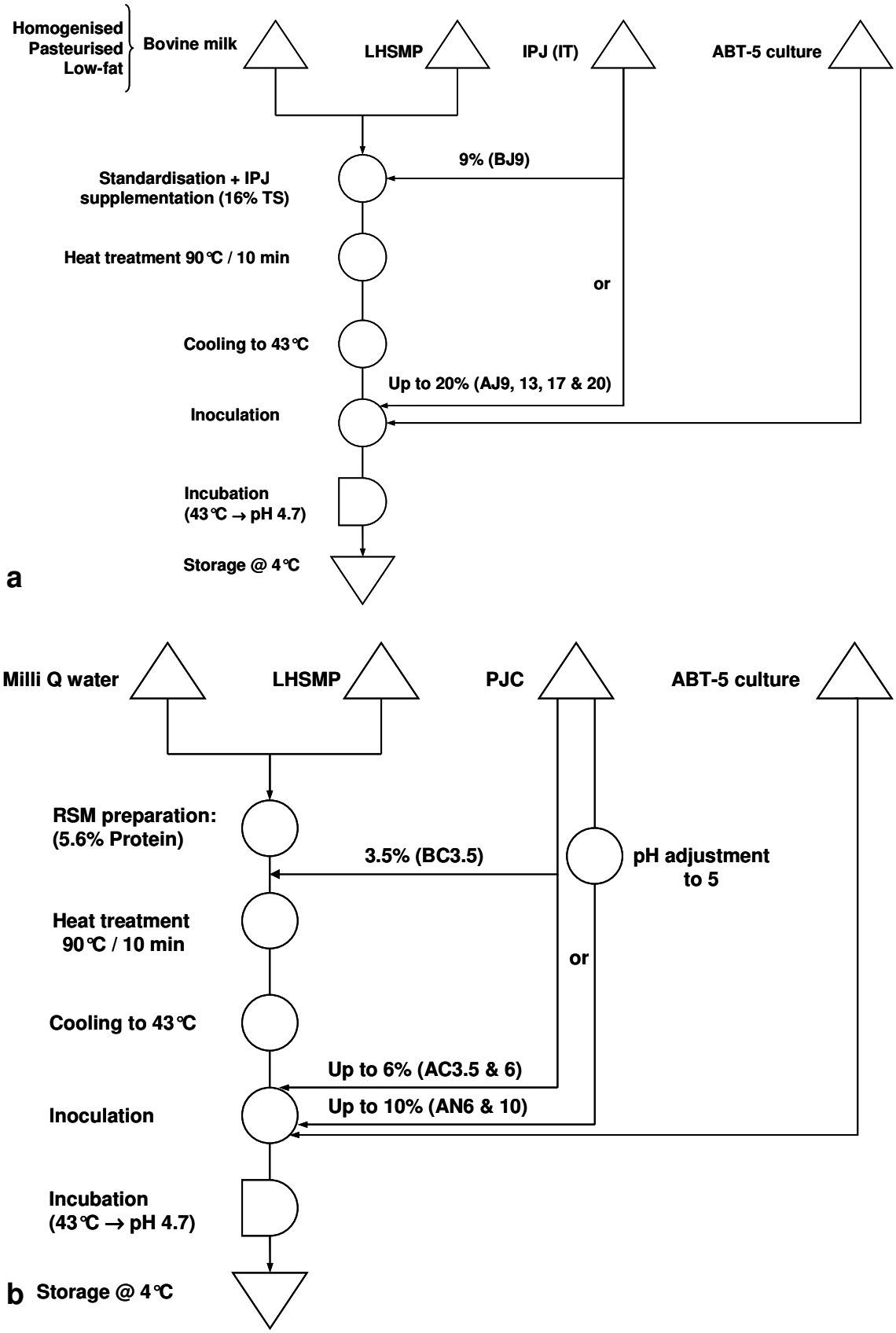


Figure 2.5 Process flow diagram of yoghurt production supplemented with (a) single strength (IPJ) and (b) concentrated pomegranate juices (PJC)

2.2.1.5 Freeze-dried yoghurt

Samples AC6 and PLR were stirred and aseptically transferred into 90 mm petri dishes to a depth of 4 to 5 mm and frozen in a blast freezer at - 28 ℃. The frozen samples were then freeze-dried in a model FDU-8612 freeze-dryer (Operon Co. Ltd, Gimpo, Korea). Once the freeze-drying cycle was complete, the yoghurt powders (FDYP) were aseptically transferred into pre-sterilised glass bottle containing silica gel pack, tightly sealed and stored at 4 ℃.

2.2.2 Physicochemical analyses

2.2.2.1 Soluble and total solids measurement

The refractive index of fresh PJ samples was determined 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 (ºB). Total solids of milk and yoghurt samples were determined using oven method according to Australian Standard (AS 2300.1.1-2008).

2.2.2.2 pH and titratable acidity (TA)

pH values of all samples were measured using a pH-meter (HI 8424, Hanna instruments, Ann Arbor, MI, USA) previously calibrated with pH 7.0 and 4.0 standard buffers. Titratable acidity of PJ samples was determined using 0.1M NaOH to the end point of pH 8.1 according the AOAC method 942.15 (AOAC 2000) and reported as % citric acid (w/v). pH and TA of milk and yoghurt samples were determined according to Australian Standards (AS 2300.1.6-2010 & AS 2300.2.10-2008).

2.2.2.3 Determination of total phenolic compounds (TPC)

Total phenolic compounds were determined by Folin-Ciocalteu (F-C) colorimetric method (Singleton and Rossi, 1965) which is based on chemical reduction of a mixture of tungsten and molybdenum oxides. This method relies on the transfer of electrons in alkaline medium from phenolic compounds to a mixture of phosphomolybdic and phosphotungstic acids to form blue complexes readable by a spectrophotometer (Ainsworth and Gillespie, 2007). Frozen juices or concentrates were thawed first, and then diluted (1:10 for PJ, and 1:25 for PJC) with Milli-Q water. Aliquots of 20 µL of diluted sample or gallic acid standard solution were transferred into 2-mL plastic cuvettes and mixed with 1.58 mL Milli-Q water. A blank was prepared using only Milli-Q water. Aliquots of 100 µL FC reagent were added to each cuvette and mixed by pipetting for *ca*. 8 min at RT (20 - 25 ºC). Then, 300 µL of 20% sodium carbonate solution was added to all cuvettes and allowed to stand for 2 h RT before reading the absorbance at 765 nm in a UV/VIS Spectrometer equipped with UV Winlab software (Lambda 35, Perkin Elmer, Waltham, MA, USA).

The freeze-dried yoghurt powder (FDYP) and yoghurt samples coded (AC6 and PLR) were diluted (1:10) in Milli-Q water and centrifuged at 18,500 g for 30 min at 4 ºC, (Eppendorf Centrifuge, 5810R, Hamburg, Germany) and 20 µL of the supernatant was used for absorbance reading as above. Whereas for yoghurt samples coded AJ9, 13, 17, 20 and PLM, the diluted (1:10 in Milli-Q water) samples were first mixed with reagents and after incubation at RT for 90 min were centrifuged as above and tested for absorbance. The absorbance reading were taken to the standard curve (Appendix 3), which was constructed from various

concentrations of gallic acid (100 to 1000 mg/L), and results were expressed as mg gallic acid equivalent (GAE) in 1 L of sample.

2.2.2.4 Determination of antioxidant activity

The antioxidant activity (AA) of PJ was measured spectrophotometrically employing ABTS method based on the generation of ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt) radical cation (Gil et al., 2000; Miller et al., 1996; Rice-Evans & Miller, 1994 and 1995; Rice-Evans et al., 1995 and 1997; Salah et al., 1995; Wolfenden and Willson, 1982).

Aliquots of 7 mM ABTS and 2.45 mM potassium persulphate aqueous solutions were mixed and kept in dark at RT for approximately 24 h until the oxidation of ABTS was complete and the absorbance stabilised. The solution containing the generated blue/green $ABTS^{\bullet+}$ chromophore radical was diluted with Milli-Q water to an absorbance of 0.70 (±0.020) at 734 nm (Re et al., 1999; Rice-Evans & Miller, 1994; Whitehead et al., 1995; Zhou et al., 2007). Two mL aliquots of $ABTS^{\bullet+}$ dilution were mixed with 200 µL aliquots of the diluted PJ (1:50 with Milli-Q water), or Trolox ® standard solution, or just Milli-Q water (as blank) in a plastic cuvette. The mixtures were allowed to stand at RT for 10 min with continuous stirring before reading the absorbance at 734 nm in a UV/VIS spectrometer. Absorbance values were taken to the standard curve (Appendix 3) prepared with Trolox solutions and results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) (Gill et al., 2000). TEAC represent the concentration (mM) of Trolox having the antioxidant capacity equivalent to 1.0 mM of the substance under investigation (Antolovich et al., 2002).

2.2.2.5 Colour measurement

Aliquots of 25 mL of commercial or fresh PJs and PJCs were transferred into disposable plastic petri dishes and the colour parameters were determined using a Chroma Meter CR-400 (Konica Minolta, Sensing, INC, Tokyo, Japan) according to the method described by Shwartz et al. (2009a & b). The readings were expressed in dimensions of L*, a*, b*, C and H°, where L* indicate the luminosity of the sample colour. Values of a*- to a*+ indicate colour change from green to red, while b*- to b*+ indicates range of colour from blue to yellow. The chroma value (C) is calculated as $C = (a^{*2} + b^{*2})^{½}$ and indicates the colour intensity or saturation. Hue angle is calculated from $H° = \tan^{-1} (b^*/a^*)$ and reflects the visual colour appearance (Solomon et al., 2006). The colour index is calculated from (180 - H°)/(L* + C) (Shwartz et al., 2009a & b; Tzulker et al., 2007). Yoghurts samples (coded AC6 and PLR) were used for colour evaluation after 24 h storage at 4 °C. The mean values of triplicate readings were reported for each sample.

2.2.2.6 Texture of yoghurt samples (large deformation analyses)

Gel firmness of set yoghurt samples coded AC6 and PLR were determined according to Amatayakul et al. (2006) and Paseephol et al. (2008) using a TA-XT2 Texture Analyser (Stable Micro System Ltd., Surry, UK) with 5 kg load cell, which was operated using software package Texture Expert Exceed (Version 1.00). A single compression test was performed using a 20 mm diameter aluminium cylindrical probe (P20) at a speed of 1.0 mm/s (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). According to Kusuma et al. (2009) adhesiveness is defined as the negative area of compression force-time curve (the first bite) representing the work necessary to pull the probe out from the yoghurt gel. Theoretically, the adhesiveness means the attractive force between surfaces of two materials.

2.2.2.7 Sensory evaluation

Samples coded SY6 and SY10 were prepared in 100 mL cups and stored overnight at 4 °C before using for organoleptic evaluation. An untrained sensory panel (n = 25) of RMIT University's Food Science students scored (Appendix 4) the yoghurt samples for aroma, colour, appearance, gel thickness, gel firmness, flavour and overall acceptability on a 10-point hedonic scale (AS 2542.1.1-2005). The samples were served immediately after removing from the fridge at 4 ºC. Panelists were asked to open the lid and evaluate aroma first and then colour and appearance by visual observation. After breaking down the yoghurt gel with a spoon and gently mixing the samples, gel thickness was rated. Finally, after placing the yoghurt in their mouth, the gel firmness, flavour and overall acceptability were evaluated.

2.2.3 Microbiological analysis

2.2.3.1 Effects of PJC on the growth of single strain cultures

The growth rate of individual lactic acid and probiotic bacteria (ST-B01, LA-5 and Bb-12) in RSMs supplemented with PJC (coded POS, POL and POB - 2.1.4.4)

was evaluated in comparison to the controls made from non-PJC supplemented RSMs (coded PLS, PLL and PLB, Table 2.2).

2.2.3.2 Effects of PJC on growth and viability of mixed cultures

The effect of PJC on the growth and viability of mixed probiotic culture ABT-5-Probio-Tec™ (a mixture of *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Streptococcus salivarius ssp. thermophilus*), was assessed over 28 days of storage at 4 °C in supplemented and control batches. Samples were prepared with the same procedure for samples coded AC6 and PLR (section 2.2.1.4.3) using heat-treated standardised RSMs. The sampling schedule for testing was 24 h post-incubation (day 1) and at weekly intervals (days 7, 14, 21 and 28). The viability of each strain in different samples was calculated according Paseephol and Sherkat (2009) using the following formula:

% Viability = (CFU/g after 4 weeks storage / initial CFU/g) $\times$ 100

2.2.3.3 Media selection

For single strain cultures, MRS, MRS-CyHCl and M17 agar were selected for enumeration of LA-5, BB-12 and ST-B01 respectively. Selective enumeration of LA-5 in mixed culture (ABT-5) was performed in MRS-sorbitol, BB-12 in MRS-NNLP and ST-B01 in M17 agar (Amatayakul et al., 2005; Dave and Shah, 1996; Shah, 1999; Rybka and Kailasapathy, 1995; Tharmaraj and Shah, 2003).

2.2.3.4 Media preparation

2.2.3.4.1 Peptone water diluent

Peptone water (0.15%) was prepared by dissolving 1.5 g of peptone water

medium in 1 L of distilled water, adjusting the pH to 7.0 ± 0.2, followed by autoclaving 90 mL and 9 mL portions at 121 ℃ for 15 min followed by immediate cooling.

2.2.3.4.2 de Man, Rogosa and Sharpe Agar (MRS agar)

MRS agar was prepared as recommended by the manufacturer by suspending 62 g of media in 1 L of distilled water. The suspension was warmed gently to boiling point to dissolve the agar, then autoclaving at 121 °C for 15 min followed by immediate cooling.

2.2.3.4.3 MRS- CyHCl

To 1 L MRS agar prepared as above, 5 mL of cystein hydrochloride (CyHCl) solution was added (55 °C) prior to use. The CyHCl solution was prepared by weighing 10 g cystein hydrochloride in a 100 mL flask and making the volume up to 100 mL with distilled water. The solution was then sterilised at 121 °C for 15 min and cooled down prior to mixing with sterile MRS agar.

2.2.3.4.4 M17 agar

M17 agar was prepared as recommended by the manufacturer by suspending 48.25 g of media in 950 mL of distilled water. The suspension was warmed gently to boiling point until the agar was dissolved, then autoclaving at 121 °C for 15 min, followed by cooling down to 50 °C before aseptically adding 50 mL of sterile 10% (w/v) lactose solution. The lactose solution was prepared by dissolving 10 g lactose in 100 mL of distilled water. The solution was then sterilised at 121 °C for 15 min and cooled down prior to use.

2.2.3.4.5 MRS-sorbitol agar

Ninety mL of MRS agar medium (section 2.2.3.4.2) was mixed with 10 mL of 10% (w/v) sorbitol solution to a final concentration of 1%. The sorbitol solution was prepared by dissolving 10 g sorbitol in 100 mL of distilled water and membrane-sterilising by passing through Millipore HA (0.45 µm) filter units (Millipore Corporation, Billerica, MA, USA).

2.2.3.4.6 MRS-NNLP

To 1 L of MRS agar medium at 50 °C (section 2.2.3.4.2) 50 mL of NNLP solution was added prior to use. The NNLP solution was prepared from 0.03 % nalidixic acid, 0.2% neomycin sulphate, 6.0% lithium chloride and 0.25% paromomycin sulphate (all w/v) in distilled water. The solution was then sterilised by passing through Millipore HA (0.45 µm) filter before adding to basal medium.

2.2.3.5 Enumeration of bacteria

Ten g of each cultured milk, FDYP or yoghurt samples was diluted with 90 mL of 0.15% sterile peptone water (section 2.2.3.4.1). Ten-fold serial dilutions (10^{-2} - 10^{-8}) were prepared in 9 mL of 0.15% sterile peptone water (AS 5013.17-2004). Based on preliminary studies, 1 mL of the last 4 dilutions was used in duplicate for enumeration using the pour plate technique. All plates were gently mixed clockwise and anticlockwise to disturb the samples uniformly and allowed to set. Duplicate plates were incubated under anaerobic condition (using Gas-pack system, AnaeroGen - 1.3) at 37 °C for 72 h, except M17 agar plates containing ST-B01 which were incubated aerobically at 37 °C for 48 h (Dave and Shah, 1996). The numbers of Colony Forming Units (CFU) on plates containing 15 to 300

colonies (AS 5013.5-2004) were calculated per gram of samples as below:

CFU g^{-1} = (Number of colonies × Volume of dilute suspension) / Dilution factor

2.2.4 Statistical analyses

All physicochemical tests were conducted in triplicate while microbial experiments were done in duplicates and the mean values ± standard deviation (SD) were reported (Appendices 6 and 7). Statistical analyses were performed by applying one-way analyses of variance (ANOVA) to determine the significance of the 95% confidence interval and correlation coefficient using Minitab software (Version 14, Minitab Inc., State College, PA, USA).

CHAPTER 3

EFFECTS OF EXTRACTION METHODS AND HEAT TREATMENT ON TOTAL PHENOLIC COPMOUNDS AND ANTIOXIDANT ACTICITY OF '*WONDERFUL*' POMEGRANATE JUICE

3.1 Physicochemical and phytochemical properties of fresh and imported pomegranate juices (IPJ)

In the first stage of this study, the fresh juices extracted from large (LPJ) and small size (SPJ) '*Wonderful*' pomegranates (section 2.2.1.1) were compared with the imported juices in terms of their physicochemical and phytochemical properties.

3.1.1 Size and yield

The average weight of the Australian pomegranates ranged between 238 ± 10 g for small fruits and 573 ± 21 g for the large ones (Section 2.1.1). The yield of arils from small pomegranates was 61.44 ± 2.11% but only 45.58 ± 2.71% from the large fruits that had thicker peels but more intensely coloured arils than the small fruits (Figure 2.1). Arils' Juice yield was comparable at 74.18 ± 3.19% and 76.54 ± 2.38% for small (SPJ) and large fruits (LPJ) respectively, however, on whole fruit basis the juice yield was expectedly lower, i.e. 45.58 ± 1.96% from small fruits and 34.89 ± 1.15% from the larger fruits.

3.1.2 Soluble solids, titratable acidity and pH

Soluble solids in LPJ was significantly (P<0.05) higher (16.8 ± 0.2 ºB) than that in SPJ (15.2 ± 0.2 ºB). In comparison, only one of the IPJs (coded UP) showed high

soluble solids content (16.1± 01 ºB) while others were significantly (P<0.05) lower ranging from 13.3 to 14.5 ºB (Table 3.1). In terms of titratable acidity, the SPJ was significantly (P<0.05) more acidic than the LPJ (1.58 ± 0.07% vs. 1.15 ± 0.09%).

Table 3.1 Chemical attributes of the fresh and imported pomegranate juices

Samples	Soluble Solids (º B)	pH	TA (% citric acid)
SPJ*	15.2 ± 0.2^{bc}	3.00 ± 0.02^{de}	1.58 ± 0.07^{ab}
LPJ*	16.8 ± 0.2^{a}	3.25 ± 0.01^{b}	1.15 ± 0.09^{cd}
IPJ (TT)	14.1 ± 0.1^{cd}	3.34 ± 0.01^{a}	1.01 ± 0.06^{de}
IPJ (TB)	14.5 ± 0.1^{c}	3.02 ± 0.01^{d}	1.61 ± 0.08^{ab}
IPJ (UP)	16.1 ± 0.1^{ab}	3.32 ± 0.01^{ab}	1.09 ± 0.05^{de}
IPJ (IT)	13.3 ± 0.1^{d}	3.15 ± 0.01^{c}	1.27 ± 0.07^{cd}

* Juice produced from small (SPJ) and large (LPJ) fruits' arils by electric juicer in two stages; IPJ: Imported pomegranate juices. Data represent the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference (P<0.05).

The acidity of SPJ was closer (P<0.05) to that of IPJ coded TB. Likewise, the acidity of the LPJ and the IPJ coded IT was not significantly different (P<0.05) (Table 3.1). While the pH levels were significantly different among the samples, the SPJ with the lowest pH (3.00 ± 0.02) was close to that of IPJ coded TB. Likewise, the pH of LPJ (3.25 ± 0.01) and IPJ coded UP were close (Table 3.1).

3.1.3 Colour parameters

The colour evaluation results of fresh and imported PJs are presented in Table 3.2. The values of L*, a*, C, Hº and colour index were not significantly different (P<0.05) between SPJ and LPJ, however, SPJ showed higher b* value than LPJ.

Table 3.2 Colour evaluation in the fresh and imported pomegranate juices

Samples	L*	a*	b*	c	Hº	Colour index	Visual appearance
SPJ*	17.50 ± 0.08^{e}	15.83 ± 0.70^{cd}	7.89 ± 0.46^{d}	17.69 ± 0.83^{de}	26.50 ± 0.36^{e}	4.36 ± 0.10^{b}	
LPJ*	17.45 ± 0.32^{e}	15.43 ± 1.50^{cd}	6.24 ± 0.58^{ed}	16.64 ± 1.60^{de}	22.05 ± 0.44^{e}	4.64 ± 0.25^{b}	
IPJ (TT)	26.67 ± 0.18^{d}	20.38 ± 0.96^{bc}	21.33 ± 0.80^{ab}	29.36 ± 1.48^{bc}	46.32 ± 0.30^{c}	2.39 ± 0.07^{d}	
IPJ (TB)	27.16 ± 0.55^{dc}	23.22 ± 2.06^{b}	17.86 ± 1.90^{b}	29.29 ± 2.79^{bc}	37.54 ± 0.54^{d}	2.53 ± 0.16^{d}	
IPJ (UP)	25.37 ± 0.99^{d}	29.07 ± 1.22^{ab}	19.56 ± 1.20^{ab}	35.04 ± 1.68^{ab}	33.92 ± 0.52^{d}	2.42 ± 0.10^{d}	
IPJ (IT)	43.18 ± 0.89^{a}	6.67 ± 0.02^{ed}	23.46 ± 0.74^{a}	24.40 ± 0.72^{cd}	74.11 ± 0.42^{ab}	1.57 ± 0.04^{e}	

* Juice produced from small size (SPJ) and large size (LPJ) fruit's arils by electric juicer in two stages; IPJ: Imported pomegranate juices. Data represent the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference (P<0.05).

The colour values of the commercial products (IPJs) were significantly different (P<0.05) to fresh juices (SPJ and LPJ), since they were all produced from concentrates. The commercial juices showed significantly higher (P<0.05) L* values (i.e. brighter), a* value (more red except for IT) and b* value (yellower) than the fresh juices. The IPJ coded IT showed a significantly higher (P<0.05) L* and b* values but the lowest a* value (Table 3.2). According to H° formula [H° = $\tan^{-1}$ (b*/a*)] an increase in the redness of a sample (a*+) or drop in its yellowness (b*+) results in low H° value that leads to increased colour index [(180 - H°)/(L* + C)] (Figure 3.1). The H° values of IPJs were significantly higher (P<0.05) and their colour indices were significantly lower than fresh juices (Table 3.2).

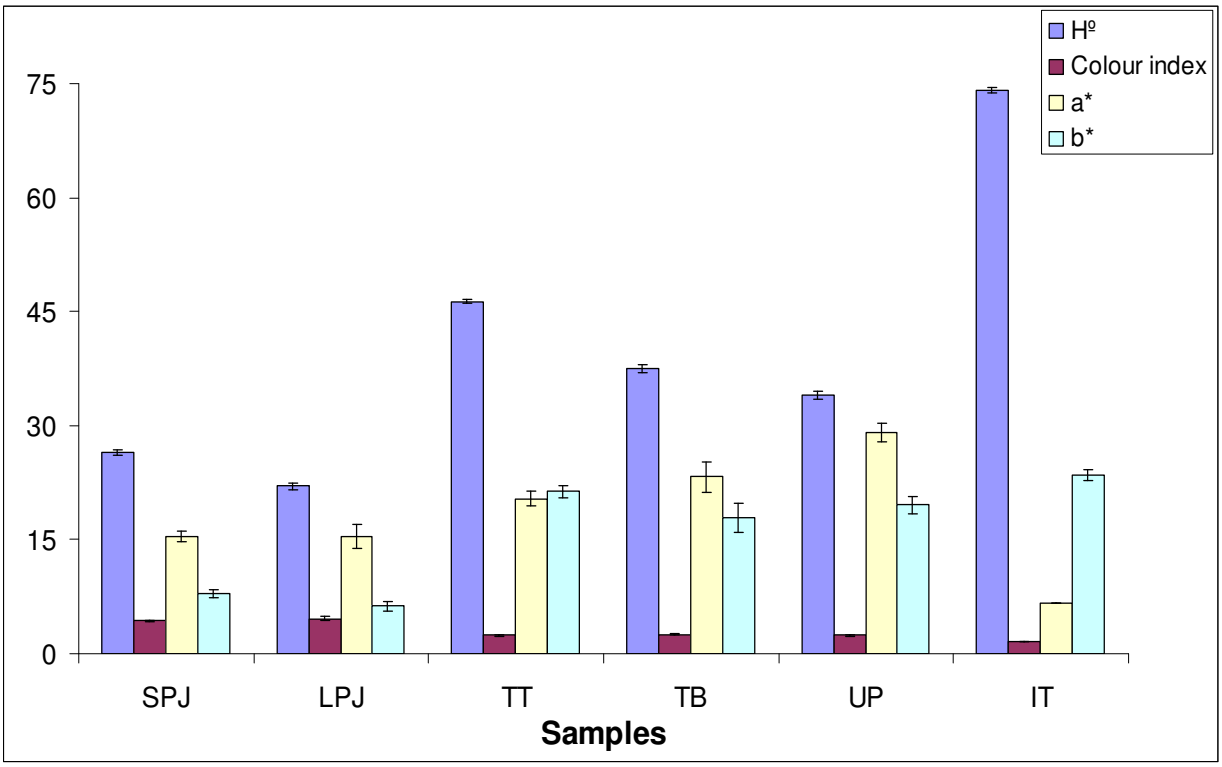


Figure 3.1 Correlation between colour parameters (Hº, a*, b* and colour index) of the fresh and imported pomegranate juices.

Data represent the means ± SD of triplicate analyses from each trial.

These results were correlated with visual appearances of samples and indicated that the colour of both SPJ and LPJ were more appealing and that the commercial products showed inferior colour compared to fresh juices (Shwartz et al., 2009a & b; Tzulker et al., 2007).

3.1.4 Phytochemical content of the fresh and imported pomegranate juices

The health benefits attributed to pomegranate fruit consumption are related, at least in part, to their antioxidant activity (AA) (Seeram et al., 2006a; Vardin & Fenercioglu, 2003). The AA of SPJ, LPJ and IPJs as determined using ABTS method (section 2.2.2.4) were found to be11.06 and 11.36 mM/L TEAC, while IPJs coded UP, TT, TB and IT showed 13.43, 9.59, 6.91 and 6.48 mM/L TEAC, respectively (Table 3.3).

Table 3.3 Total phenolic compounds and antioxidant activity in the fresh and imported pomegranate juices

Samples	TPC (GAE mg/L)	AA (TEAC mM/L)
SPJ*	2460 ± 164^{ab}	11.06 ± 0.91^{bc}
LPJ*	2545 ± 97^{ab}	11.36 ± 0.94^{ab}
IPJ (TT)	1923 ± 177^{bc}	9.59 ± 0.93^{bc}
IPJ (TB)	1293 ± 113^{cd}	6.91 ± 0.97^{cd}
IPJ (UP)	2630 ± 245^{ab}	13.43 ± 0.99^{ab}
IPJ (IT)	1193 ± 171^{d}	6.48 ± 0.90^{bc}

* Juice produced from small size (SPJ) and large size (LPJ) fruit's arils by electric juicer in two stages; IPJ: Imported pomegranate juices. Data represent the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference (P<0.05).

In pomegranates like other fruits such as blueberry, black cherry, cranberry or red wine the level of AA can be mainly attributed to the level of total phenolic compounds (Gil et al., 2000; Seeram et al., 2008; Solomon et al., 2006; Tzulker et al., 2007; Zhuang et al., 2011). Therefore, the TPC in PJs were measured by F-C method (section 2.2.2.3) and the results were expressed as mg/L GAE (Table 3.3).

Freshly extracted juices SPJ and LPJ showed close TPC levels, i.e. 2,460 and 2,545 mg/L GAE, respectively; whereas the IPJs (UP, TT, TB and IT) showed quite a vast range of TPC, namely 2,630, 1,923, 1,293 and 1,193 mg/L GAE, respectively (Table 3.3). Gil et al. (2000) suggested that the industrial extraction process either increases the amount of TPC or enhances the activity of the antioxidants. Industrially, the whole fruit is pressed hydrostatically which results in the extraction of large amounts of polyphenols from the peels (Tzulker et al., 2007). The juice is then filtered, concentrated and bulk-stored. The juice bottling companies extend the concentrate with water to desired concentration then pack and distribute it. Thus the level of dilution determines the TPC level and the AA of the commercial juices.

Among the IPJs tested in this study, only sample coded UP showed a comparable TPC level to fresh aril juice while other samples failed in this regard. These results are in agreement with the soluble solids (SS) of samples tested, although SS alone could not be a reliable indicator of AA activity, since some IPJs may have added sugar or blended with other cheaper juices to adjust their SS to an acceptable level.

This clearly confirms that the antioxidant activity of pomegranate juices is mainly due

to its TPC level. Thus, any improvement in extraction process aimed at increasing the level of TPC in the juice should have a direct effect on the antioxidant activity.

3.2 Effects of raw materials and extraction method on physicochemical and phytochemical properties of pomegranate juice

In the second stage of this study different parts of the fruit were used for juice extraction using six different extraction methods (section 2.2.1.1) to improve the TPC levels in the fresh PJs. The resulting juices were coded PJ1 to PJ6 and their physicochemical and phytochemical attributes were determined.

3.2.1 Yield

The yields (calculated on whole fruit base) of PJs 1, 2, 3 and 5 were not significantly (P<0.05) different (Table 3.4). In regards to the yields of PJs 1 and 2 (produced from arils), double extraction with electric juicer (PJ1) produced similar yield as PJ2 produced using manual pressing. However, when these two methods were combined (as in PJs 4 and 6) the juice yield increased significantly (P<0.05).

3.2.2 Soluble solids, titratable acidity and pH

The type of fruit fraction and the extraction methods used showed direct effect on the soluble solids content of all samples. Accordingly, soluble solids in PJs 1 and 2 (15.2 ± 0.2 °B) produced from arils with similar yields were not significantly (P<0.05) different. Due to differences in the fruit parts used for extraction, the soluble solids in PJ5 was significantly (P<0.05) higher (16.4 ± 0.2) than that in PJ3 (15.9 ± 0.1), whereas PJ6 had significantly (P<0.05) more soluble solids (16.9 ± 0.1) than PJ4 (16.1 ± 0.1). While, based on extraction method employed, soluble solids in PJ6 was

significantly (P<0.05) higher than that in PJ5, which in turn was significantly (P<0.05) higher than that in PJ4 (Table 3.4).

The pH of PJs 1 and 2 extracted from arils were not significantly different (P<0.05) but when non-edible piths or peels of the fruit were included in PJ extraction the level of pH increased significantly (P<0.05) to ca. 3.10. This was also evident in the level of titratable acidity in PJs 1 and 2 (1.58 ± 0.07 and 1.64 ± 0.10 respectively) that were more acidic than PJs 3 to 6 which were not significantly different (P<0.05) to each other (Table 3.4).

Table 3.4 Effects of the raw materials and extraction methods on the yield and chemical properties of pomegranate juices

Samples	Yield	Soluble solids (° B)	pH	TA (% citric acid)
PJ1	45.58 ± 1.96^{bc}	15.2 ± 0.2^{d}	3.00 ± 0.02^{de}	1.58 ± 0.07^{abc}
PJ2	45.04 ± 2.06^{bc}	15.2 ± 0.2^{d}	3.00 ± 0.01^{de}	1.64 ± 0.10^{ab}
PJ3	45.23 ± 2.07^{bc}	15.9 ± 0.1^{c}	3.08 ± 0.01^{bc}	1.32 ± 0.07^{cd}
PJ4	56.87 ± 1.64^{a}	16.1 ± 0.1^{bc}	3.10 ± 0.01^{b}	1.42 ± 0.09^{bcd}
PJ5	40.04 ± 2.04^{bc}	16.4 ± 0.2^{bc}	3.10 ± 0.01^{ab}	1.36 ± 0.07^{cd}
PJ6	49.78 ± 2.12^{cd}	16.9 ± 0.1^{ab}	3.10 ± 0.02^{b}	1.38 ± 0.09^{cd}

Data shown represents the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference (P<0.05).

3.2.3 Colour parameters

Differences in raw materials and extraction methods affected the colour parameters of the extracted juices. The correlations between these parameters are presented in Table 3.5. No significant differences (P<0.05) were found between the L* values of PJs 1 and 5, PJs 2 and 3, and PJs 4 and 6 and between the a* values of PJs1 and 3, PJs 5 and 6 and between the b* values of PJs 4 and 5. PJ1 showed the highest H° value (26.50 ± 0.36) that declined as the extraction method became more extensive, the lowest value being that of PJ6 (14.13 ± 0.96). The colour index was not affected by the type of raw materials used, and in contrast with H° values, the highest and lowest colour indices were observed in PJ6 (4.68 ± 0.19) and PJ1 (4.36 ± 0.10) respectively. These results were correlated with visual appearances of samples and in agreement with Shwartz et al. (2009a & b) and Tzulker et al. (2007) who indicated that the samples with higher colour indices had strong red appearances.

3.2.4 Improving total phenolic compounds and antioxidant activity by changing raw materials and extraction intensity

Incorporation piths and peels and increasing the extraction intensity (section 2.2.1.1) increased the TPC levels. In juices extracted from arils, the TPC in PJ1 (2460 ± 164 mg/L GAE) was significantly higher than PJ2 (2071 ± 62 mg/L GAE) due to difference in extraction method used (2-satges vs. manual), but the AA of these samples were not statistically different (P<0.05) (Table 3.6). When peeled and chopped fruit pieces were used as raw material, more TPC was found in the juice resulting in higher AA than juice from separated arils. As the extraction method became more intensive significantly (P<0.05) higher TPC were extracted leading to even more

Table 3.5 Colour evaluation in pomegranate juices extracted using different fruit parts and different methods

Samples	L*	a*	b*	c	Hº	Colour index	Visual colour
PJ1	17.50 ± 0.08^{cd}	15.83 ± 0.70^{bc}	7.89 ± 0.46^{a}	17.69 ± 0.83^{abc}	26.50 ± 0.36^{a}	4.36 ± 0.10^{bcd}	
PJ2	18.50 ± 0.15^{ab}	15.44 ± 1.92^{bcd}	7.47 ± 0.21^{ab}	16.97 ± 2.08^{abc}	24.54 ± 0.37^{ab}	4.39 ± 0.27^{bcd}	
PJ3	18.42 ± 0.32^{ab}	15.97 ± 0.77^{bc}	5.86 ± 0.23^{bc}	17.01 ± 0.79^{abc}	20.17 ± 0.34^{bc}	4.51 ± 0.14^{bc}	
PJ4	18.09 ± 0.04^{bc}	13.37 ± 1.74^{cd}	4.76 ± 0.52^{cd}	17.20 ± 1.34^{abc}	19.61 ± 0.41^{c}	4.55 ± 0.18^{bc}	
PJ5	17.75 ± 0.20^{cd}	17.45 ± 1.14^{abc}	4.86 ± 0.35^{cd}	18.12 ± 1.19^{ab}	15.56 ± 0.48^{d}	4.59 ± 0.15^{abc}	
PJ6	17.88 ± 0.03^{bc}	17.06 ± 1.68^{abc}	4.28 ± 0.12^{d}	17.59 ± 1.65^{abc}	14.13 ± 0.96^{de}	4.68 ± 0.19^{abc}	

Data represent the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference (P<0.05).

antioxidant activity. E.g. PJ4 with 7293 ± 605 mg/L GAE and 30.25 ± 2.10 mM/L TEAC *cf* PJ3 with 5760 ± 609 mg/L GAE and 23.13 ± 2.88 mM/L TEAC (Table 3.6).

Upon using unpeeled chopped whole fruit pieces for juice extraction the TPC level and AA of PJs 5 and 6 were further improved compared to all other samples (Table 3.6), however, the difference between PJs 5 and 6 in terms of the TPC content (11545 ± 503 *cf* 12516 ± 167 mg/L GAE) or the antioxidant activity (50.65 ± 1.60 *cf* 56.91 ± 2.79 mM/L TEAC) was not statistically significant ($P<0.05$).

Table 3.6 Total phenolic and antioxidant activity in juice samples extracted from different parts of the fruit using different extraction methods

Samples	TPC (GAE mg/L)	AA (TEAC mM/L)
PJ1	2460 ± 164^{de}	11.06 ± 0.91^{d}
PJ2	2071 ± 62^{e}	9.16 ± 0.97^{d}
PJ3	5760 ± 609^{cd}	23.13 ± 2.88^{c}
PJ4	7293 ± 605^{c}	30.25 ± 2.10^{bc}
PJ5	11545 ± 503^{ab}	50.65 ± 1.60^{a}
PJ6	12516 ± 167^{ab}	56.91 ± 2.79^{a}

Data represent the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference ($P<0.05$).

Over five fold increase in TPC and AA level could be achieved in PJs by combining different extraction methods with chopped whole fruits. These results support previously reported works (Cam et al., 2009; Drogoudi and Tsipouridis,

2005; Gil et al., 2000; Shwartz et al., 2009a & b) and confirm that the AA of different samples was directly linked with the level of TPC in those samples (Figure 3.2).

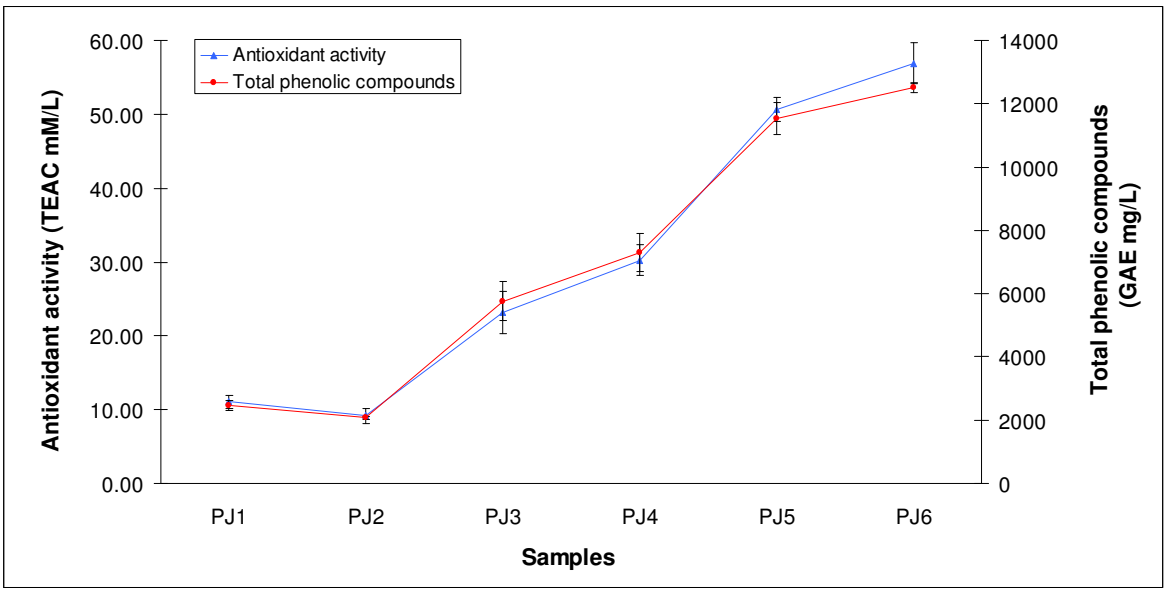


Figure 3.2 The relationship between the levels of antioxidant activity (TEAC mM/L) and total phenolic compounds (GAE mg/L) in juice samples.

Data represent the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference (P<0.05).

3.3 Effects of juice pasteurisation on total phenolic compounds and antioxidant activity

Fresh Juice samples (PJs 1 to 6) were pasteurised individually at 90 ºC for 15 sec (section 2.2.1.2) and the resulting samples were coded PJ1P to PJ6P. The TPC level in pasteurised PJs 1P (2293 ± 248 mg/L GAE), 3P (5297 ± 764 mg/L GAE) and 5P (10456 ± 472 mg/L GAE) were 6.8, 8.0 and 9.4% lower than unpasteurised PJs 1 (2460 ± 164 mg/L GAE), 3 (5760 ± 609 mg/L GAE) and 5 (11545 ± 503 mg/L GAE) respectively, while in PJ2P, PJ4P and PJ6P the TPC level remained unchanged (P<0.05) after heat treatment (Figure 3.3). These results confirmed

that the pasteurisation regime employed in this project did not have a serious impact on TPC levels or the AA of all samples (Figure 3.3).

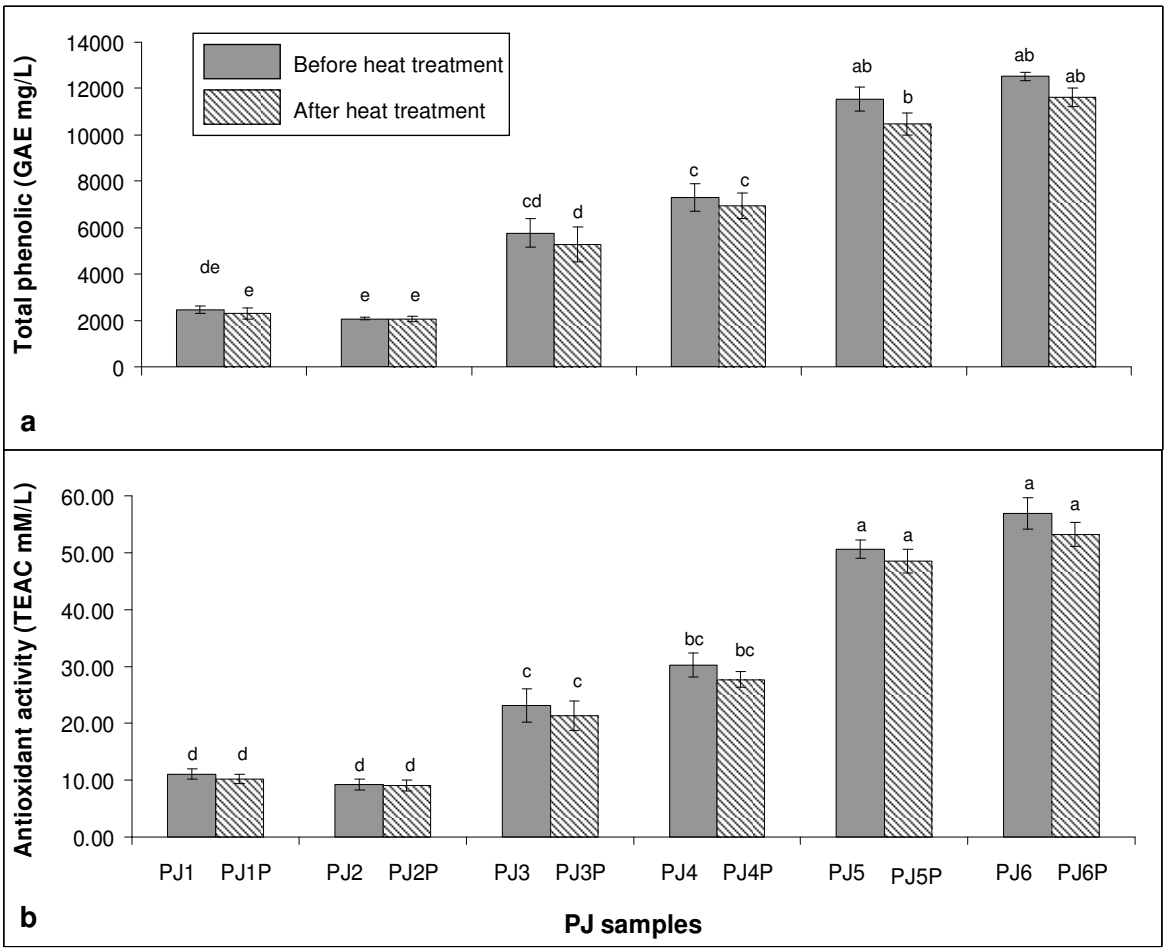


Figure 3.3 Effects of pasteurisation on (a) total phenolic compounds (GAE mg/L) and (b) antioxidant activity (TEAC mM/L) of fresh juice samples.

Data represent the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference (P<0.05).

The physicochemical and phytochemichal properties of the Australian '*Wonderful*' pomegranates grown in Robnivale (Victoria) were determined and compared with 4 different brands of imported commercial pomegranate juices. The fresh fruits were grouped in two size classes. The larger fruits seemed to have lower juice

yield, however, their juice was slightly less sour and more sweet with higher SS, and contained over 3.3% more TPC and nearly 2.7% higher AA than juice from smaller fruits. The results indicated that the TPC and AA of the freshly extracted Juices were higher than three of the four imported commercial PJs tested in this project while the sample coded UP had comparable levels of TPC and AA to fresh juice. These differences could be due to varietal differences, processing conditions or simply the dilution effect.

Different extraction procedures were combined with various raw materials - arils, peeled and whole fruits - to improve the TPC level and AA in PJs. The results indicated that TPC level and AA can be increased six times in PJs extracted from based whole fruit.

CHAPTER 4

PROBIOTIC AND ANTIOXIDANT PROPERTIES OF PROBIOTIC YOGHURT SUPPLEMENTED WITH POMEGRANATE JUICE

4.1 Preliminary studies on probiotic yoghurt

4.1.1 Probiotic yoghurt Production

A probiotic product was developed by incorporating PJ in the formulation of probiotic yoghurt (section 2.2.1.4.2). The IPJ coded IT was used in the preliminary studies. Different levels of PJ (9, 13, 17 and 20%) were added to standardised milk before or after heat treatment at 90 ℃ for 10 min. Supplementation before heat treatment was limited to 9%, since above this level the milk curdled, whereas after heat treatment, up to 20% IPJ could be added without any adverse effect. It appeared that heat treatment of milk increased its stability and buffering capacity. By increasing the level of supplementation to 20% the pH of milk before incubation declined to 5.56 (Figure 4.1). After inoculating with probiotic culture (ABT-5) the samples were incubated at 43 ℃ to reach pH 4.7. At this pH the time was recorded and yoghurt samples were transferred to refrigerated storage at 4 ℃.

The activity of the starter culture in the preliminary study was estimated from changes in time to reach target pH (Figure 4.1). Comparing the yoghurt setting times in 17 and 20% (*ca.* 5 h) and in 9 and 13 % (*ca.* 6 h) supplemented samples it was noted that PJ could have an influence on the activity of starter culture. The indepth study of the impact of PJ on the activity and survival of lactic acid and probiotic bacteria is reported in section 4.2.4.

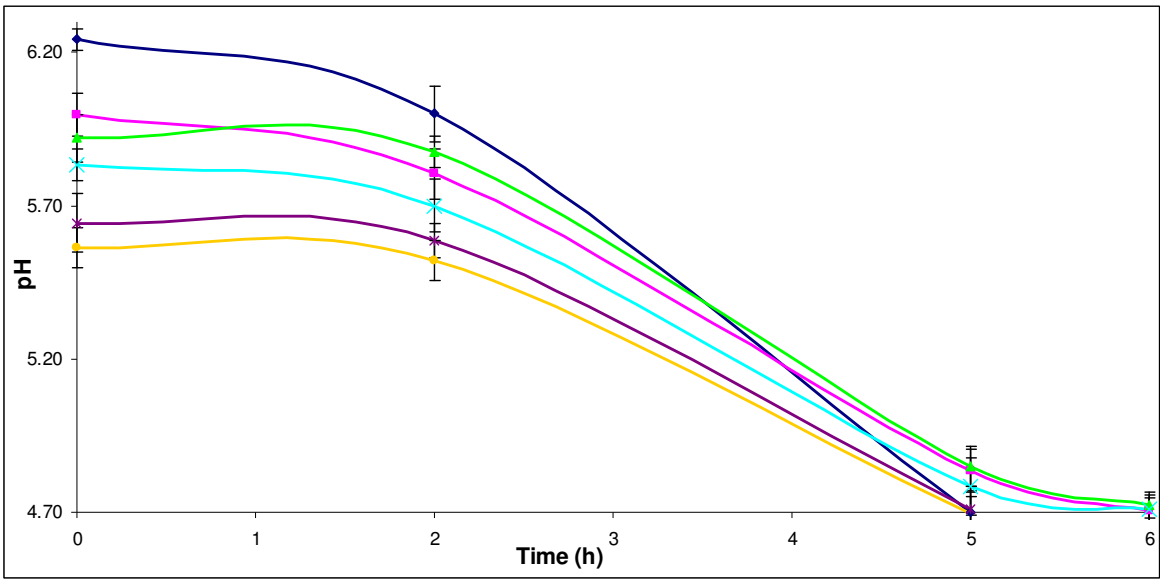


Figure 4.1 pH change in yoghurts supplemented with pomegranate juice before or after heat treatment.

The data represent the means ± SD of triplicate analyses from each trial.

- ◆ Plain yoghurt (PLM)
- ■ +9% PJ before heat treatment (BJ9)
- ▲ +9% PJ after heat treatment (AJ9)
- × +13% PJ after heat treatment (AJ13)
- × +17% PJ after heat treatment (AJ17)
- ● +20% PJ after heat treatment (AJ 20)

4.1.2 Determination of total phenolic compounds in probiotic yoghurt

The Folin-Ciocalteu (F-C) colorimetry method (section 2.2.2.3) was used to determine the total phenolic compounds (TPC) of the supplemented yoghurt samples. In the preliminary tests diluted yoghurt samples (coded AJ9, 13, 17, 20, BJ9 and PLM) were mixed with reagents and after incubation (RT for 90 min) were centrifuged (18,500 g for 30 min at 4 ºC) and tested for absorbance as described in section 2.2.2.3.

The TPC of probiotic yoghurts containing 9% (BJ9) pomegranate juice (before heat treatment) and 9 (AJ9), 13 (AJ13), 17 (AJ17) and 20% (AJ20) (after heat

treatment) was found to be 585, 583, 637, 688 and 731 mg/L GAE, respectively while the background TPC in plain yoghurt (PLM) was 514 mg/L GAE and in pure PJ (IT) was 1193 mg/L GAE (Figure 4.2).

To eliminate the interference of yoghurt components in the reaction, the diluted yoghurt sample (1:10 in Milli-Q water) was first centrifuged (as above) and the supernatant was used for the assay with F-C method as mentioned in section 2.2.2.3. With this change in sample preparation, the obtained results for plain yoghurt made from RSM coded PLR (1153 ± 32 mg/L GAE) became comparable to that of Sonmez et al. (2010) who reported the TPC of plain UHT milk measured with F-C method as 1030 ± 19 mg/L GAE.

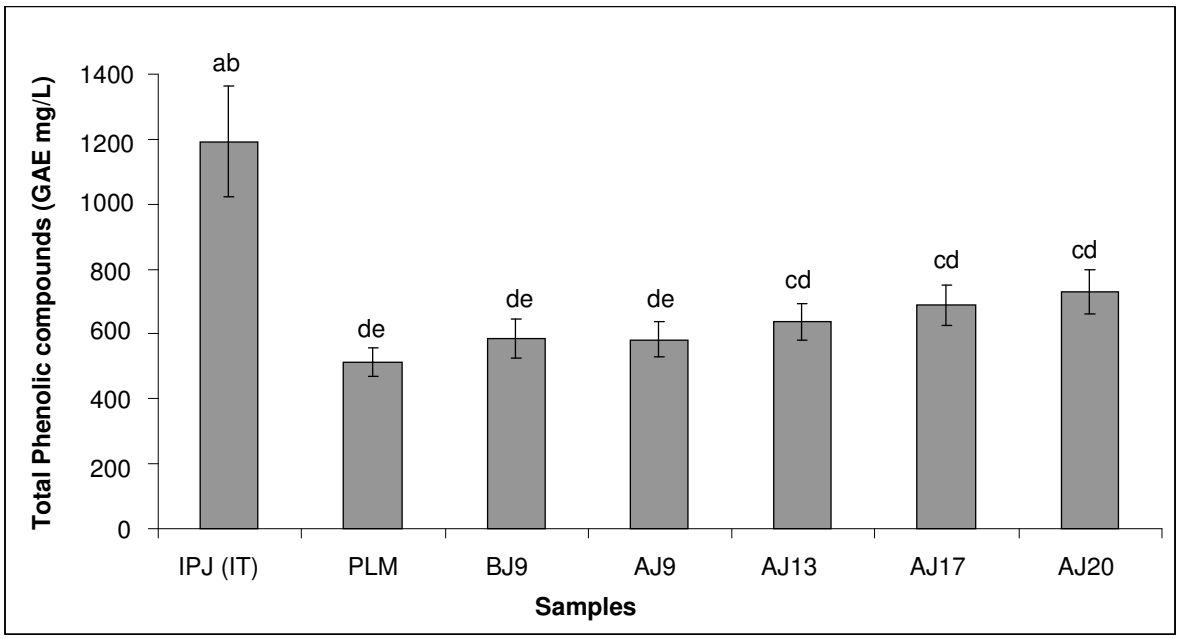


Figure 4.2 Total phenolic compounds (mg/L GAE) in IPJ (coded IT) and in yoghurts supplemented with it at different levels.

Data represent the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference ($P<0.05$).

4.2 Yoghurt supplementation with pomegranate juice concentrate

The concentrated PJ1P (52 ºB) with its higher amount of TPC (7483 ± 73) and soluble solid was used in the formulation of probiotic yoghurt (section 2.2.1.4.3) to eliminate the dilution affect of the straight juice. Probiotic yoghurt was supplemented with PJC at 3.5% and 6% level before and after heat treatment. In agreement with preliminary studies results (section 4.1.1) heat treatment of RSM increased its stability and the buffering capacity of milk proteins. Supplemented samples were then inoculated with probiotic culture (ABT-5) and incubated at 43 ºC to reach to pH 4.7.

During the incubation time the activity of the starter culture was monitored by measuring the pH (Figure 4.3). Supplementation with 3.5% PJC before and after heat treatment dropped the milk pH to 5.96 and 5.81 before incubation, while 6% supplementation level further decreased the pH to 5.70. The control yoghurt reached the target pH after 5 h while the supplemented samples showed 1 h delay reaching to target pH after 6 h. 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 effects PJC on the activity and survival of these bacteria (section 4.2.4).

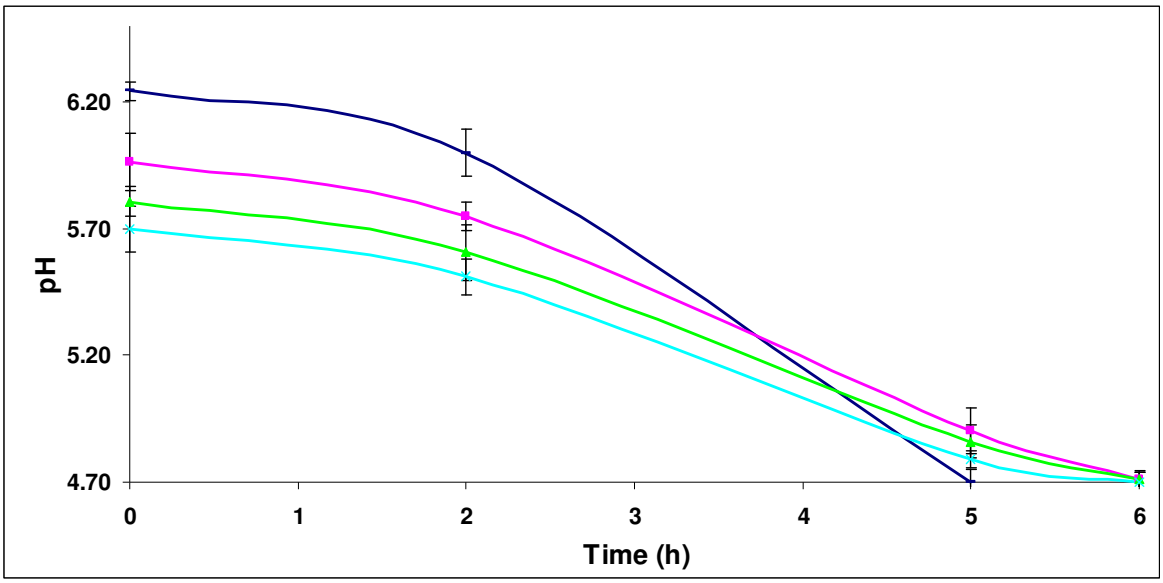


Figure 4.3 pH change in yoghurts supplemented with pomegranate juice concentrate. The data represent the means ± SD of triplicate analyses from each trial.

- Plain yoghurt (PLR)
- ■ +3.5% PJC before heat treatment (BC3.5)
- ▲ +3.5% PJC after heat treatment (AC3.5)
- × +6% PJC after heat treatment (AC6)

4.2.1 Effects of Pomegranate juice concentrate addition on total phenolic compounds level in probiotic yoghurt

The TPC of the PJ concentrate was found to be 7483 mg/L GAE. Six percent of this concentrate was added to milk used for yoghurt production (coded AC6, section 2.2.1.4.3). As a result the polyphenols level increased from 1153 ± 32 mg/L GAE in the control yoghurt (coded PLR) to 1590 ± 34 mg/L GAE In supplemented yoghurt (AC6).

4.2.2 Effects of pomegranate juice concentrate addition on colour parameters of probiotic yoghurt

As expected, all colour parameters (Table 4.1) were significantly (P<0.05) different between control (PLR) and supplemented sample (AC6). Addition of PJC with its low L* (19.31 ± 0.22), b* (1.13 ± 0.05), c (3.61 ± 0.08) and H° (18.15 ± 0.42) and high a* (13.43 ± 0.08) and colour index (7.06 ± 0.07) 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 -5.17 to +6.45. At the same time, the luminosity (L*) and colour intensity (c) of samples decreased significantly (P<0.05). According to H° formula [$H° = \tan^{-1} (b^*/a^*)$] any increase in the redness of a sample (a*+) or drop in its yellowness (b*+) results in low H° value. The H° value of AC6 (36.83) was significantly (P<0.05) lower than PLR (112.22), while its colour index value was significantly (P<0.05) higher (1.71 vs. 0.64). These results correlated with visual observation of the samples and were in agreement with obtained results in sections 3.1.3 and 3.2.3 and indicated that the sample with higher colour index (AC6) had pink appearances.

Table 4.1 Changes in colour parameters of yoghurt by the addition of pomegranate juice concentrate

Sample	L*	a*	b*	c	H°	Colour index
PLR	92.67 ± 0.12^{a}	- 5.17 ± 0.07^{e}	12.65 ± 0.12^{a}	13.67 ± 0.14^{ab}	112.22 ± 0.14^{a}	0.64 ± 0.00^{d}
AC6	74.99 ± 0.35^{de}	6.45 ± 1.04^{ab}	5.28 ± 0.12^{e}	8.80 ± 0.03^{de}	36.83 ± 0.84^{e}	1.71 ± 0.01^{a}

Data represent the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference ($P<0.05$).
PLR: Control yoghurt made from RSM and ABT-5; AC6: Same yoghurt supplemented with 6% PJC after heat treatment; PJC: Pomegranate juice concentrate

4.2.3 Effects of pomegranate juice concentrate addition on texture of probiotic yoghurt

Table 4.2 presents the results of texture analyses performed on probiotic yoghurt supplemented with PJC (AC6) and the control sample (PLR) using large deformation analyses (section 2.2.2.6) on day one. Supplementation with PJC (AC6) significantly reduced the fracture force by 0.22 (N), firmness by 0.11 (N) and adhesiveness by 1.34 (N.s) in comparison with PLR. However, despite the inferior textural attributes the supplemented sample was still considered acceptable (Figure 4.4). Texture of yoghurt is influenced by various factors such as quality and composition of milk and its protein and fat content, heat treatment, combination of lactic acid bacteria used, acidification rate, and storage time (Dello Staffolo et al., 2004; Purwandari et al., 2007; Sodini et al., 2004). Protein content and type is the most important factor influencing textural properties of yoghurt. Higher protein content would cause a higher degree of cross-linking of the gel network resulting in a firmer gel structure (Paseephol et al., 2008). In preparation of both yoghurt batches (PLR and AC6) the amount of protein in milk was kept constant by the addition of appropriate amount of LHSMP (section

Table 4.2 Changes in textural attributes of yoghurt as a result of pomegranate juice concentrate addition

Samples	Fracture force (N)	Firmness (N)	Adhesiveness (N.s)
PLR	1.49 ± 0.02^{ab}	1.78 ± 0.10^{abcd}	6.13 ± 0.09^{a}
AC6	1.27 ± 0.03^{de}	1.67 ± 0.09^{bcde}	4.97 ± 0.05^{de}

Data represent the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference ($P<0.05$).
PLR: Control yoghurt from RSM and ABT-5; AC6: Same yoghurt supplemented with 6% PJC after heat treatment

2.2.1.4.3). It is possible that the PJC upon dispersion in the milk serum and interfered with the protein matrix formation thus resulting in a softer yoghurt gel in the supplemented sample.

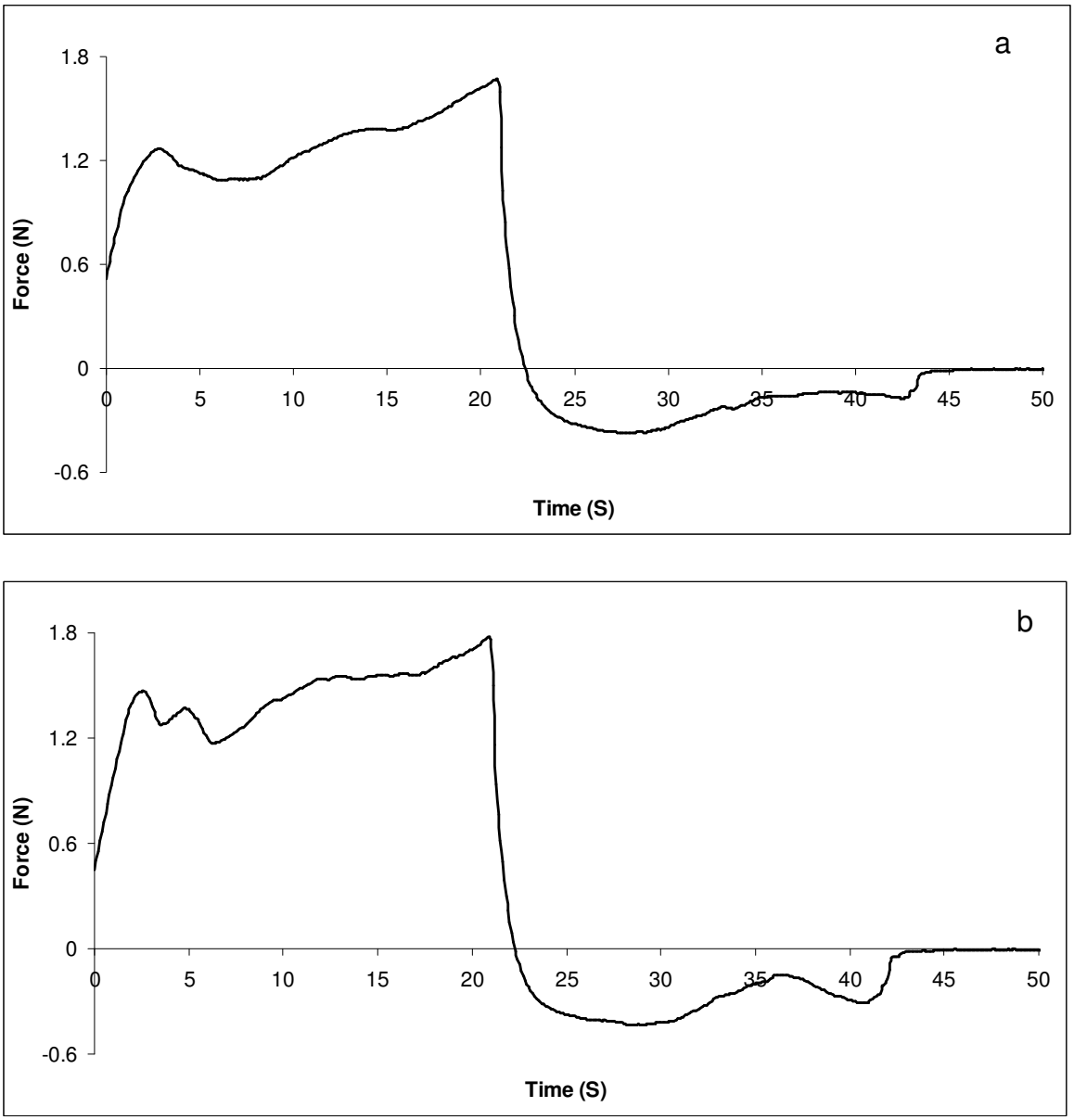


Figure 4.4 Comparative large deformation analysis of (a) probiotic yoghurt made by RSM and ABT-5 supplemented with 6% PJC (AC6) and (b) non-supplemented (control) sample (PLR) made by RSM and ABT-5

4.2.4 The impact of pomegranate juice concentrate addition on the growth and viability of probiotic and lactic acid bacteria in yoghurt

Two different models were designed (section 2.2.1.4.3 and 4) to monitor the effects of PJC addition on the survival of *Lactobacillus acidophilus* (LA-5), *Bifidobacterium bifidum* (BB-12) and *Streptococcus salivarius ssp. thermophilus* (ST-B01). In the first model, the effects of PJC supplementation on the viable count of mixed probiotic bacteria and starter culture in ABT-5 was studied, while, in the second model the viability of single strain of LA-5, BB-12 and ST-B01 were evaluated in PJC-supplemented sample against the control.

The means of replicate experiments were determined according to Australian standard (AS 5013.5-2004) stating that the absolute difference between two independent single test results obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, should not be greater than the repeatability limit, $r = 0.25$ in $\log_{10}$ microorganisms per mL. Accordingly, the difference between the first and the second set of results with a probability level of 95 % was not greater than 0.25 $\log_{10}$ units.

4.2.4.1 Evaluation of the viable count of the mixed probiotic and lactic acid bacteria in yoghurt during refrigerated storage

Changes in viable counts of ST-01, LA-5 and BB-12 in the control (PLR) and PJC supplemented (AC6) yoghurts from day 1 to the end of the shelf life of 28 days is presented in Table 4.3.

Table 4.3 Variations in the viable counts of ST-B01, LA-5 and BB-12 in control (PLR) and PJC-supplemented (AC6) yoghurts during storage at 4 ℃

Culture	Period (day)	PLR (log CFU/g)	AC6 (log CFU/g)
ST-B01	1	7.47 ± 0.16^{abc}	7.20 ± 0.13^{ab}
	7	7.36 ± 0.12^{bcd}	7.17 ± 0.26^{ab}
	14	7.26 ± 0.07^{cde}	7.21 ± 0.16^{ab}
	21	7.42 ± 0.14^{bcd}	7.20 ± 0.13^{ab}
	28	7.38 ± 0.08^{bcd}	6.80 ± 0.15^{cd}
	% Viability	98.83^{AB}	94.48^{DE}
LA-5	1	6.72 ± 0.16^{ab}	6.65 ± 0.17^{ab}
	7	6.35 ± 0.09^{cd}	6.36 ± 0.13^{bcd}
	14	6.43 ± 0.16^{cd}	6.42 ± 0.12^{bc}
	21	6.30 ± 0.11^{cde}	6.20 ± 0.16^{cd}
	28	6.27 ± 0.10^{de}	6.03 ± 0.17^{de}
	% Viability	93.23^{AB}	90.61^{CD}
BB-12	1	6.58 ± 0.21^{abc}	6.47 ± 0.17^{abc}
	7	6.49 ± 0.15^{abc}	6.38 ± 0.14^{bc}
	14	6.41 ± 0.15^{bcd}	6.29 ± 0.10^{bcd}
	21	6.32 ± 0.09^{bcd}	6.23 ± 0.06^{cd}
	28	6.28 ± 0.10^{cd}	6.06 ± 0.10^{de}
	% Viability	95.52^{ABCD}	93.70^{BCDE}

The data represent the means ± SD of duplicate experiments with duplicate tests. ANOVA was used to determine the significance of differences at a confidence level of 0.05 as identified by different letters (lower case in the same column in each section and upper case in the same row).
% Viability = (CFU/g after 4 weeks storage/initial CFU/g) × 100.
PLR: Control yoghurt from RSM and ABT-5
AC6: Same yoghurt supplemented with 6% PJC added post-heat treatment

At the beginning of storage (day 1) ST-B01 numbers were over 7 log CFU/g in both yoghurts while the initial population of LA-5 ranged from 6.65 (AC6) to 6.72 (PLR) log CFU/g and BB-12 from 6.47 (AC6) to 6.58 (PLR) log CFU/g. After 7

days the number of ST-B01 in control sample (PLR) decreased by 0.2 log cycle and did not significantly (P<0.05) change during the storage period (Table 4.3) Supplementation with PJC decreased the number of ST-B01 for *ca.* 0.2 log cycle in day 1 which did not changed significantly (P<0.05) up to day 21, but by the last week of storage, its final count was *ca.* 0.58 log cycle less than control sample.

The number of LA-5 decreased significantly (P<0.05) in both control and supplemented samples in day 7 and demonstrated a steady and slow decline for the following two weeks. These results reveal that the PJC supplementation had low effect on population of LA-5 up to week 3 (*ca.* 0.1 log cycle). In the last week, the population of LA-5 in control batch dropped only by 0.03 log cycle, while in yoghurt containing PJC (AC6) the drop was more noticeable (*ca.* 0.17 log cycle) and in the final count showed the *ca.* 0.2 log cycle less than the control sample.

The population of BB-12 did not significantly (P<0.05) change up to day 7 in control sample (PLR). Although demonstrated slow decline during the second week it did not change significantly (P<0.05) during the third week and showed marginal decline of 0.3 log cycle in the last week to 6.28 log CFU/g. Supplementation with PJC decreased the number of BB-12 for *ca.* 0.1 log cycle on the first day of storage, followed a steady decline until the end of storage period when the final counts dropped to 6.06 log CFU/g, i.e. 0.2 log cycle less than the control sample.

The viability of lactic and probiotic cultures during the yoghurt shelf life declined significantly (P<0.05) due to PJC supplementation (Table 4.3). The retention of

viability of ST-B01 (98.83% for PLR and 94.48% for AC6) was better than those for LA-5 (93.23% for PLR and 90.61% for AC6) and BB-12 (95.52% for PLR and 93.70% for AC6). This observation was consistent with the findings of Akalin et al. (2004), Dave and Shah (1996), Medina and Jordano (1994), Ozer et al. (2005) and Paseephol and Sherkat (2009) who reported higher stability of ST-B01 than LA-5 and BB-12 in probiotic yoghurts during storage time.

4.2.4.2 Viability of single strains of LA-5, BB-12 and ST-B01 in model systems

To further clarify the effects of PJC supplementation on the individual strains of LA-5, BB-12 and ST-B01 a model was designed as described in section 2.2.1.4.4. Changes in viable counts of ST-B01, LA-5 and BB-12 in the controls (PLS, PLL and PLB) and PJC supplemented sterilised RSM models (POS, POL and POB) after 12 h of incubation at 37 ºC are presented in Table 4.4.

Table 4.4 The viable counts of Lactic acid and probiotic bacteria in the control and PJC-supplemented model systems

Test system	ST-B01 (log CFU/g)	LA-5 (log CFU/g)	BB-12 (log CFU/g)
Controls (PLS,PLL,PLB)	6.77 ± 0.14 ^{abcd}	7.59 ± 0.14 ^{abcd}	7.38 ± 0.10 ^{ab}
Models (POS,POL,POB)	6.76 ± 0.16 ^{abcd}	7.60 ± 0.11 ^{abcd}	6.39 ± 0.13 ^{de}

The data represent the means ± SD of duplicate experiments, and each experiment was examined in duplicate. The different letters in each column show a significant difference ($P<0.05$).
PLS, PLL and PLB: RSM fermented with ST-B01, LA-5 or BB-12, respectively
POS, POL and POB: RSM supplemented with 6% PJC and fermented with ST-B01, LA-5 or BB-12

PJC addition did not significantly (P<0.05) affect the population of ST-B01 and LA-5 in single strain models after overnight incubation, however, the count of BB-12 decreased significantly (P<0.05).

By comparing the effects of PJC on the viability of mixed starter culture (Table 4.3) and single strains (Table 4.4), it was noted that although the viable count of BB-12 decreased significantly (P<0.05) in single strain model (1.01 log CFU/g), in the mixed culture its loss of viability was less than other bacteria. This may be due to possible synbiotic-type interactions among the bacteria in the mixed culture.

4.3 Sensory evaluation of probiotic yoghurts supplemented with pomegranate juice concentrate

As described in section 4.2 the supplementation level of PJC in the formulation of probiotic yoghurt was limited to 6% since above this level the milk curdled. To increase the level of supplementation, the pH of PJC was first adjusted with NaOH (7N) to a final pH of 5 and then used in the formulation of probiotic yoghurt at 6 (SY6) and 10% (SY10) levels (section 2.1.4.3). At this higher pH the maximum level of PJC supplementation was limited to 10% due to change in milk attributes. Adjustment of pH to higher than 5 changed the PJC taste that was not acceptable.

Samples SY6 and SY10 were subjected to sensory evaluation (section 2.2.2.7) and evaluated for their aroma, colour, appearance, gel thickness and firmness, flavour and overall acceptability. The average sensory scores of the untrained panelists (n=25) are presented in Table 4.5.

Addition of 4% more PJC in the formulation of sample SY10 compared to sample SY6 affected all the sensory attributes except the gel thickness that was not significantly (P<0.05) different to SY6 although the texture was slightly firmer (Figure 4.5). One of the most obvious effects of PJC supplementation was the development of grainy texture in yoghurt which was more obvious in SY10. This could be the result of interaction between milk protein and the acids of PJC during incubation.

As expected due to higher PJC level in sample SY10, panelists were able to perceive stronger fruity aroma and flavour with deeper pink colour (Figure 4.5). Overall acceptance scores revealed that sample SY10 was significantly (P<0.05) more desirable to the panelists than sample SY6.

Results obtained indicated that although sample SY10 presented slightly grainy and firmer gel, its deeper pink colour and more fruity aroma and flavour resulted in higher acceptability among the panelists in comparison to sample SY6 (Figure 4.5).

Table 4.5 Sensory attributes of probiotic yoghurts supplemented with 6 and 10% pomegranate juice concentrate

Samples	Aroma	Colour	Appearance	Gel thickness	Gel firmness	Flavour	Overall acceptability
SY6*	4.64 ± 1.38^{bcd}	3.80 ± 1.22^{de}	7.08 ± 1.15^{abc}	6.60 ± 1.15^{bcd}	4.52 ± 1.19^{bcd}	3.48 ± 1.87^{de}	3.96 ± 1.51^{de}
SY10**	5.08 ± 0.95^{abc}	6.92 ± 1.73^{ab}	6.48 ± 1.16^{bcd}	6.68 ± 1.03^{bcd}	5.12 ± 1.39^{abc}	6.64 ± 1.85^{abc}	6.92 ± 1.93^{ab}

* 6% supplementation; ** 10% supplementation; n=25
The data represent the means ± SD of triplicate analyses from each trial. The different letters in each column show a significant difference (P<0.05).

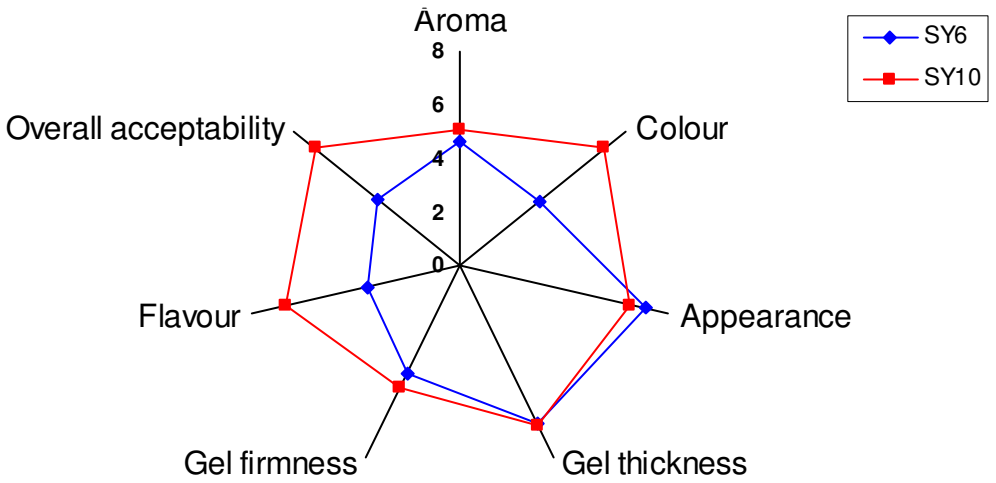


Figure 4.5 Polar presentation of sensory data (n=25)

4.4 Effects of freeze-drying on probiotic yoghurts bacteria and total phenolic compounds

The PJs 5 and 6 extracted from unpeeled chopped whole fruits (section 2.2.1.1) showed high level of TPC (section 3.2.4) compared to other extracted juices. The extensive extraction method used resulted in PJs with strong astringency and limited their usage for direct consumption. One of the possible ways of their usage could be as a concentrate in formulation of freeze-dried yoghurt powder (FDYP) that could be used as a food supplement in capsule form. Although in this project we did not produce PJs 5 and 6 capsules, but the effects of freeze-drying on the probiotic yoghurt (AC6) were assessed.

Sample AC6 was subjected to freeze-drying as described in section 2.2.1.5. The resulting FDYP was further analysed for the viable counts (log CFU/g) of ST-B01, LA-5 and BB-12 and total phenolic compounds (GAE mg/L).

Freeze-drying of yoghurt and cultured dairy products has been described by many researchers (Capela et al., 2006; Kim and Bhowmik, 1990; Pan et al., 1994; Rybka and Kailasapathy, 1995). During freeze-drying, the frozen water is removed by sublimation, thus avoiding heat damage to biological structures (Capela et al., 2006). Freeze-dried yoghurt can be stored for up to 1-2 years at 4 ℃. This process not only preserves yoghurt but also helps maintain a sufficient quantity of viable probiotics in the product (Kumar and Mishara, 2004).

Changes in viable counts and TPC level of the control and PJC-supplemented FDYPs at the first day of their production are presented in Table 4.6.

Table 4.6 Viable counts of lactic acid and probiotic bacteria and total phenolic compounds in freeze-dried yoghurt powders

Sample & calculation	ST-B01 (log CFU/g)	LA-5 (log CFU/g)	BB-12 (log CFU/g)	TPC (GAE mg/L)
Control FDYP	8.25 ± 0.04^{abc}	7.40 ± 0.12^{abc}	7.31 ± 0.11^{bcd}	7119 ± 56^{d}
Calculated*	8.29^{ab}	7.45^{ab}	7.36^{abc}	7183^{d}
Supplemented FDYP	8.01 ± 0.05^{de}	7.29 ± 0.07^{cd}	7.21 ± 0.11^{de}	8327 ± 48^{a}
Calculated*	8.04^{cde}	7.34^{bcd}	7.25^{cde}	8284^{a}

Each experiment was examined in duplicate (microbial analysis) and triplicate analyses from each trial (TPC analysis). The data represent the means ± SD. The different letters in each column show a significant difference (P<0.05).
*Calculated count = CFU/g before freeze drying × (100/ yield)
*Calculated TPC = TPC before freeze drying × (100/ yield)

Considering the yields of freeze-drying for control and supplemented samples (16.06 ± 0.01% and 19.20 ± 0.02%) the viable counts of bacteria and TPC are also calculated and presented in Table 4.6.

The TPC in control and supplemented FDYPs were found to be higher than that of fresh yoghurt samples (coded PLR and AC6) used for freeze-drying (*ca.* 7183 vs. 1153 mg/L GAE and 8327 vs. 1590 mg/L GAE). These results were not significantly (P<0.05) different from the calculated amounts and revealed that freeze-drying procedure did not adversely affect the TPC content in FDYPs.

Removing the frozen water by sublimation during the freeze-drying increased the concentration of lactic acid and probiotic bacteria in both control and supplemented FDYPs. The population of ST-B01, LA-5 and BB-12 in

supplemented FDYP were increased by 0.81, 0.64 and 0.74 log cycle compared to fresh probiotic yoghurt (AC6) used for freeze-drying. These results (Table 4.6) showed marginal decline in bacterial population in both control and supplemented samples in comparison to the calculated amounts, which were in agreement with Capela et al, (2006) who showed a slight negative effect of freeze-drying on bacteria viability in FDYPs.

The effects of supplementation level and stage of supplementation on yoghurt attributes were analysed. The results indicated that up to 20% PJ and 6% PJC could be supplemented post-heat treatment, with no adverse effect on the cultures' activity or yoghurt quality. The probiotic yoghurt supplemented with 6% PJC showed high TPC level (ca. 1590 ± 34 mg/L GAE) and more than 90% viability of lactic and probiotic cultures after 28 days storage at 4 ºC.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

To improve the AA and TPC level in PJs different parts of pomegranate fruits were used and subjected to different extraction methods. Up to six fold increase in TPC level could be achieved in samples extracted from whole fruits. While, strong astringency of these juices could limit their applications for direct consumption, they may however find uses in nutraceuticals as AA supplements. The results also indicated that pasteurisation (90 ºC for 15 sec) did not significantly (P<0.05) affect the AA of the extracted PJs, although TPC levels of PJ1, PJ3 and PJ5 declined after heat treatment.

In the research and development part, the probiotic yoghurt product was developed by incorporating PJ and PJC as a supplement. The effects of different percentages of PJ and PJC supplementations in different stages of yoghurt making procedure were analysed. The results indicated, up to 20% PJ and 6% PJC could be supplemented post-heat treatment, and no apparent antagonism was observed between the cultures' activity and the PJ phytochemicals during the incubation time.

Further analyses on the probiotic yoghurt supplemented with PJC indicated that although it showed inferior textural properties compared to control sample but it had higher colour index and contained over 37% more TPC (*ca.*1590 mg/L GAE). Considering the recommended polyphenols intake of *ca*. 1 g/day (Baghurst, 2006) this probiotic product offers a pleasant and effective route to increasing the

antioxidant intake in our daily diet.

The microbiological study on the individual lactic and probiotic organisms (ST-B01, LA-5 and BB-12) revealed that PJC supplementation decreased the number of BB-12 in single strain model system but not so for ST-B01 and LA-5. The interaction among these bacteria in mixed culture resulted in more than 90% viability of lactic and probiotic cultures after 28 days storage at 4 ºC.

The results of sensory evaluation (n=25) on the probiotic yoghurt supplemented with 6 and 10% pH-adjusted PJC revealed that, by increasing the level of supplementation the overall acceptability among the panellists increased significantly ($P<0.05$).

To analyse the effects of freeze-drying on the TPC and population of lactic and probiotic bacteria in supplemented yoghurt, sample AC6 was subjected to freeze-drying. Obtained results revealed that, although the freeze-drying showed the slight negative effect on viability of bacteria but it did not on TPC. Removing water from probiotic yoghurt by freeze-drying increased the amount of TPC (5.2 times) and the population of probiotic bacteria by 0.64 (LA-5) and 0.74 (BB-12) log cycle in FDYP.

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

1. Based on the literature, different environmental conditions could affect PJ attributes. This study analysed the physicochemical and phytochemichal properties of the '*Wonderful*' pomegranates grown in Robivale (Victoria) but further study on the '*Wonderful*' variety from different climatic zones of Australia is essential.

2. Considering the high TPC and AA of PJs 5 and 6 and their strong astringency, further investigations are needed to use them as AA supplements in other functional foods.

3. New formulation of probiotic yoghurt supplemented with PJs 5 and 6 (i.e. fresh or freeze-dried or microencapsulated powder) need for further investigation.

4. To overcome the textural problems of the developed probiotic yoghurt, more investigation is needed on the formulation and processing of yoghurt (such as using sodium casienate or carageenan in yoghurt formulation).

5. The effects of 10% pH-adjusted PJC supplementation on the probiotic yoghurt attributes (TPC and probiotic viability) needs further investigation.

6. The next phase of this study could involve animal or human feeding trials to substantiate the health benefits of the probiotic product developed in this project.

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APPENDICES

Appendix 1 Traditional pomegranate products

Anardana

The conventional utilisation of wild pomegranate fruits is mainly in the form of dried arils, which is called pomegranate raisins or ‘‘Anardana’’ (Kingsly and Singh, 2007). Anardana has a distinct tart flavour, and is commercially available in many Asian countries, where they are consumed in large quantities. Anardana is also used in the ayurvedic medicine as digestive and stomachic. Jaiswal et al. (2010) studied Anardana’s anthocyanins and evaluated the effects of different heat treatment on the availability of these compounds during drying procedure. Kingsly and Singh (2007) and Singh et al. (2007) evaluated the effects of different separation techniques and drying methods on pomegranate arils and the quality of Anardana.

Traditional pomegranate juice concentrate

Orak (2009) analysed the AA, TPC, colour and nutritional attributes of PJ and its traditional concentrate produced by atmospheric evaporation in 50 kg vessel for 8 h. The AA and TPC in the concentrate were found to be higher than that of PJ. They indicated that the reducing sugars, glucose and fructose level and the amount of potassium and magnesium increased during the traditional concentration method.

Appendix 2 Development of pomegranate-based new products

Pomegranate and lemon juice blend

Gonzalez-Molina et al. (2009) produced new polyphenol-rich beverages by blending lemon juice (LJ) and PJ in different proportions (25% LJ : 75% PJ; 50% LJ : 50% PJ and 75% LJ : 25% PJ v/v) and studied their bioactive composition (flavonoids and Vit. C), the AA and colour stability over a 70-day storage period. The results suggested that the blend containing 75% PJ and 25% LJ had high antioxidant activity due its phenolic composition - punicalagin isomers, anthocyanins and Vit. C - and improved colour properties.

Pomegranate juice encapsulation

Robert et al. (2010) encapsulated the PJ (obtained from fruits grown in 'Las Cardas' experimental station of the University of Chile, Ovalle, Chile) and its ethanolic extracts with maltodextrin and soy protein isolates by spray drying. The stability of polyphenols and anthocyanins in microcapsules was studied at 60 °C for 56 days. The polyphenols encapsulating efficiency was significantly better in soy protein isolates matrix whereas for anthocyanins better efficiency was achieved in maltodextrin matrix. The microcapsules were subsequently added to yoghurt samples and the stability of polyphenols was studied for 56 days at 4 °C. The polyphenols degradation rate of microcapsules was the same as the non-encapsulated PJ, except for PJ ethanolic extracts encapsulated with maltodextrin which was significantly (P<0.05) lower than the other samples.

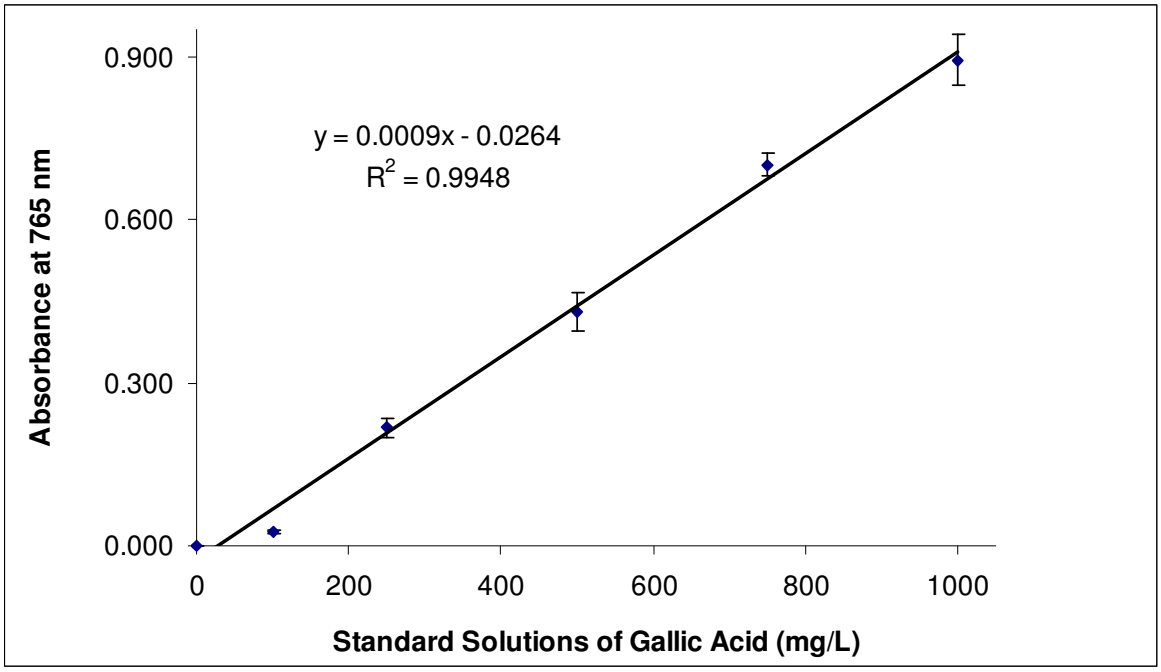
Fermented pomegranate juice and cold press seed oil

The AA, TPC and eicosanoid enzyme inhibition properties of fermented PJ (with wine yeast, *Saccharomycs bayanus*, at room temperature for 10 days) and seed oil flavonoids (mixed cultivars from the Neve Yaar research station, Israel) were studied by Schubert et al. (1999). They also analysed the fatty acid composition of cold pressed seed oil. The fermented PJ and cold pressed seed oil showed strong AA close to that of butylated hydroxyanisole (BHA) and green tea, and significantly higher than that of red wine.

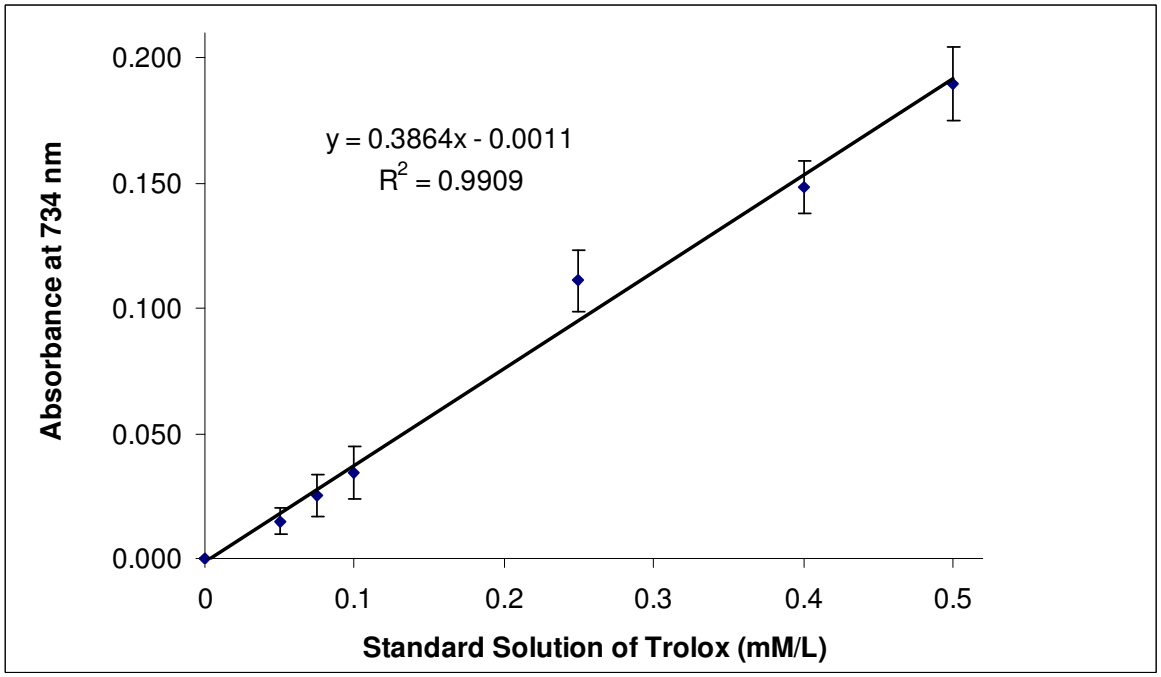
Mousavi et al. (2011) investigated the suitability of pomegranate juice as a non-dairy probiotic drink. They fermented PJ by four strains of lactic acid bacteria: *Lactobacillus plantarum*, *L. delbruekii*, *L. paracasei*, *L. acidophilus* at 30 ℃ for 72 h under microaerophilic conditions. They analysed the microbial population, pH, titrable acidity, sugar and organic acid metabolism during the fermentation period and also the viability of all strains during the storage at 4 ℃ for 4 weeks. Their results showed better microbial growth for *L. plantarum* and *L. delbruekii* during fermentation. They indicated that PJ could be a suitable media for production of a fermented probiotic drink.

Appendix 3 Standard curves

(a)



(b)



Appendix 4 Questionnaire for sensory evaluation of yoghurt samples

Instruction: You are given two different pomegranate yoghurt samples coded PL and PJ. Please evaluate them on the ten points 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 yoghurt gel with spoon and gently mix the samples to evaluate the yoghurt thickness. After placing product in your mouth evaluate the gel firmness and flavour.

- **Aroma**

Plain Yoghurt — Fruit yoghurt

PL ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

PJ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

1 2 3 4 5 6 7 8 9 10

- **Colour**

Light pink — Deep pink

PL ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

PJ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

1 2 3 4 5 6 7 8 9 10

- **Appearance**

Very grainy — Not grainy

PL ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

PJ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

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

PL ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

PJ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

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
PL	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
PJ	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	1	2	3	4	5	6	7	8	9	10

- **Flavour:** Fruity sensation

	Weak fruity									Strong fruity
PL	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
PJ	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	1	2	3	4	5	6	7	8	9	10

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

	Like least									Like most
PL	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
PJ	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	1	2	3	4	5	6	7	8	9	10

Additional comments on aroma, flavour or texture of samples:

Appendix 5 Triplicate results of chapter 3

Table 5.1 The average weight of small and large size pomegranate fruits

Samples	Weight (g)			Ave	STD
	1	2	3		
Small size	227	241	247	238	10
Large size	551	576	593	573	21

Table 5.2 The yield of arils from small and large size pomegranates

Samples	Arils yield (%)			Ave	STD
	1	2	3		
Small size	59.25	61.61	63.46	61.44	2.11
Large size	42.84	45.62	48.27	45.58	2.71

Table 5.3 The Juice yield arils basis for small and large size pomegranates

Samples	Juice yield % (arils basis)			Ave	STD
	1	2	3		
Small size (SPJ)	71.83	72.91	77.81	74.18	3.19
Large size (LPJ)	74.55	75.89	79.18	76.54	2.38

Table 5.4 The Juice yield whole fruits basis for small and large size pomegranates

Samples	Juice yield % (whole fruits basis)			Ave	STD
	1	2	3		
Small size	44.13	44.80	47.81	45.58	1.96
Large size	33.65	35.10	35.91	34.89	1.15

Table 5.5 Yield of pomegranate juices extracted with different methods

Samples	Juice yield % (whole fruits basis)			Ave	STD
	1	2	3		
PJ1	44.13	44.80	47.81	45.58	1.96
PJ2	43.08	44.84	47.19	45.04	2.06
PJ3	42.96	45.71	47.02	45.23	2.07
PJ4	55.05	57.34	58.23	56.87	1.64
PJ5	37.70	41.46	40.96	40.04	2.04
PJ6	47.72	49.66	51.96	49.78	2.12

Table 5.6 Soluble solids (º B), pH and titratable acidity - TA (% citric acid) in Australian and imported pomegranate juices

Samples	Soluble solids (º B)			Ave	STD	pH			Ave	STD	TA (% citric acid)			Ave	STD
	1	2	3			1	2	3			1	2	3		
SPJ	15.0	15.2	15.4	15.2	0.2	3.00	3.02	2.98	3.00	0.02	1.59	1.51	1.65	1.58	0.07
LPJ	16.8	16.6	17.0	16.8	0.2	3.26	3.24	3.25	3.25	0.01	1.06	1.23	1.15	1.15	0.09
IPJ (TT)	14.2	14.0	14.2	14.1	0.1	3.34	3.33	3.34	3.34	0.01	0.94	1.03	1.05	1.01	0.06
IPJ (TB)	14.6	14.4	14.6	14.5	0.1	3.01	3.03	3.03	3.02	0.01	1.52	1.64	1.66	1.61	0.08
IPJ (UP)	16.2	16.0	16.2	16.1	0.1	3.32	3.31	3.32	3.32	0.01	1.10	1.13	1.03	1.09	0.05
IPJ (IT)	13.4	13.2	13.4	13.3	0.1	3.15	3.14	3.16	3.15	0.01	1.22	1.25	1.35	1.27	0.07

Table 5.7 Soluble solids (º B), pH and titratable acidity - TA (% citric acid) of pomegranate juices extracted with different methods

Samples	Soluble solids (º B)			Ave	STD	pH			Ave	STD	TA (% citric acid)			Ave	STD
	1	2	3			1	2	3			1	2	3		
PJ1	15.0	15.2	15.4	15.2	0.2	3.00	3.02	2.98	3.00	0.02	1.59	1.51	1.65	1.58	0.07
PJ2	15.2	15.0	15.4	15.2	0.2	3.01	3.00	2.99	3.00	0.01	1.61	1.75	1.55	1.64	0.10
PJ3	16.0	15.8	16.0	15.9	0.1	3.07	3.08	3.08	3.08	0.01	1.26	1.29	1.40	1.32	0.07
PJ4	16.2	16.2	16.0	16.1	0.1	3.09	3.10	3.10	3.10	0.01	1.34	1.39	1.52	1.42	0.09
PJ5	16.4	16.2	16.6	16.4	0.2	3.11	3.10	3.10	3.10	0.01	1.28	1.39	1.41	1.36	0.07
PJ6	17.0	16.8	16.8	16.9	0.1	3.11	3.08	3.10	3.10	0.02	1.33	1.32	1.48	1.38	0.09

Table 5.8 Colour evaluation in Australian and imported pomegranate juices (L^*, a^* and b^*)

Samples	L^*			Ave	STD	a^*			Ave	STD	b^*			Ave	STD
	1	2	3			1	2	3			1	2	3		
SPJ	17.41	17.56	17.52	17.50	0.08	16.63	15.54	15.32	15.83	0.70	8.42	7.62	7.64	7.89	0.46
LPJ	17.79	17.38	17.17	17.45	0.32	17.14	14.77	14.37	15.43	1.50	6.91	5.86	5.96	6.24	0.58
IPJ (TT)	26.72	26.81	26.47	26.67	0.18	20.99	20.88	19.27	20.38	0.96	21.79	21.79	20.41	21.33	0.80
IPJ (TB)	27.05	27.75	26.67	27.16	0.55	24.72	24.07	20.87	23.22	2.06	19.17	18.74	15.68	17.86	1.90
IPJ (UP)	25.07	26.47	24.56	25.37	0.99	30.06	29.44	27.71	29.07	1.22	20.57	19.88	18.23	19.56	1.20
IPJ (IT)	43.99	42.23	43.32	43.18	0.89	6.70	6.66	6.66	6.67	0.02	24.30	23.21	22.88	23.46	0.74

Table 5.9 Colour evaluation in Australian and imported pomegranate juices (c, Hº, colour index)

Samples	c			Ave	STD	Hº			Ave	STD	Colour index			Ave	STD
	1	2	3			1	2	3			1	2	3		
SPJ	18.64	17.30	17.12	17.69	0.83	26.85	26.13	26.51	26.50	0.36	4.25	4.41	4.43	4.36	0.10
LPJ	18.48	15.89	15.55	16.64	1.60	21.96	21.66	22.53	22.05	0.44	4.36	4.76	4.81	4.64	0.25
IPJ (TT)	30.26	30.18	27.65	29.36	1.48	46.08	46.22	46.65	46.32	0.30	2.35	2.35	2.46	2.39	0.07
IPJ (TB)	31.28	30.50	26.10	29.29	2.79	37.79	37.91	36.92	37.54	0.54	2.44	2.44	2.71	2.53	0.16
IPJ (UP)	36.42	35.52	33.17	35.04	1.68	34.38	34.03	33.35	33.92	0.52	2.37	2.35	2.54	2.42	0.10
IPJ (IT)	25.21	24.15	23.83	24.40	0.72	74.58	73.98	73.76	74.11	0.42	1.52	1.60	1.58	1.57	0.04

Table 5.10 Colour evaluation of pomegranate juices extracted with different methods (L^*, a^* and b^*)

Samples	L^*			Ave	STD	a^*			Ave	STD	b^*			Ave	STD
	1	2	3			1	2	3			1	2	3		
PJ1	17.41	17.56	17.52	17.50	0.08	16.63	15.54	15.32	15.83	0.70	8.42	7.62	7.64	7.89	0.46
PJ2	18.66	18.37	18.47	18.50	0.15	17.15	13.36	15.82	15.44	1.92	7.68	7.45	7.27	7.47	0.21
PJ3	18.06	18.51	18.69	18.42	0.32	15.10	16.55	16.25	15.97	0.77	5.60	5.95	6.03	5.86	0.23
PJ4	18.13	18.09	18.06	18.09	0.04	11.50	13.68	14.94	13.37	1.74	4.20	4.85	5.22	4.76	0.52
PJ5	17.83	17.53	17.90	17.75	0.20	18.69	17.22	16.45	17.45	1.14	5.26	4.62	4.70	4.86	0.35
PJ6	17.86	17.87	17.91	17.88	0.03	18.77	17.00	15.42	17.06	1.68	4.38	4.30	4.15	4.28	0.12

Table 5.11 Colour evaluation of pomegranate juices extracted with different methods (c, Hº, colour index)

Samples	c			Ave	STD	Hº			Ave	STD	Colour index			Ave	STD
	1	2	3			1	2	3			1	2	3		
PJ1	18.64	17.30	17.12	17.69	0.83	26.85	26.13	26.51	26.50	0.36	4.25	4.41	4.43	4.36	0.10
PJ2	18.79	14.71	17.41	16.97	2.08	24.12	24.80	24.69	24.54	0.37	4.16	4.69	4.33	4.39	0.27
PJ3	16.11	17.59	17.33	17.01	0.79	20.35	19.78	20.37	20.17	0.34	4.67	4.44	4.43	4.51	0.14
PJ4	17.25	18.51	15.83	17.20	1.34	20.07	19.51	19.26	19.61	0.41	4.52	4.38	4.74	4.55	0.18
PJ5	19.42	17.83	17.10	18.12	1.19	15.72	15.02	15.95	15.56	0.48	4.41	4.67	4.69	4.59	0.15
PJ6	19.27	17.54	15.97	17.59	1.65	13.14	14.20	15.06	14.13	0.96	4.49	4.68	4.87	4.68	0.19

Table 5.12 Total phenolic (GAE mg/L) and antioxidant activity (TEAC mM/L) in Australian and imported juice samples

Samples	GAE mg/L			Average	STD	TEAC mM/L			Average	STD
	1	2	3			1	2	3		
SPJ	2316	2427	2638	2460	164	11.14	10.11	11.92	11.06	0.91
LPJ	2438	2571	2627	2545	97	10.75	10.88	12.44	11.36	0.94
IPJ (TT)	1804	1838	2127	1923	177	10.36	8.55	9.85	9.59	0.93
IPJ (TB)	1171	1316	1393	1293	113	6.35	8.04	6.35	6.91	0.97
IPJ (UP)	2360	2693	2838	2630	245	14.51	12.56	13.21	13.43	0.99
IPJ (IT)	1004	1238	1338	1193	171	7.00	7.00	5.45	6.48	0.90

Table 5.13 Total phenolic (GAE mg/L) and antioxidant activity (TEAC mM/L) in juice samples extracted with different procedures (before heat treatment)

Samples	GAE mg/L			Average	STD	TEAC mM/L			Average	STD
	1	2	3			1	2	3		
PJ1	2316	2427	2638	2460	164	11.14	10.11	11.92	11.06	0.91
PJ2	2004	2082	2127	2071	62	9.72	8.04	9.72	9.16	0.97
PJ3	5260	5582	6438	5760	609	24.73	19.81	24.86	23.13	2.88
PJ4	6649	7382	7849	7293	605	32.10	30.68	27.96	30.25	2.10
PJ5	10982	11704	11949	11545	503	49.31	50.22	52.42	50.65	1.60
PJ6	12349	12516	12682	12516	167	54.23	56.69	59.80	56.91	2.79

Table 5.14 Total phenolic (GAE mg/L) and antioxidant activity (TEAC mM/L) in juice samples extracted with different procedures (after heat treatment)

Samples	GAE mg/L			Average	STD	TEAC mM/L			Average	STD
	1	2	3			1	2	3		
PJ1P	2093	2216	2571	2293	248	9.59	9.98	11.14	10.24	0.81
PJ2P	1949	2049	2193	2064	123	8.68	8.29	10.11	9.03	0.95
PJ3P	4438	5538	5860	5279	746	21.88	18.52	23.69	21.36	2.63
PJ4P	6449	6804	7538	6930	555	28.87	26.15	28.09	27.70	1.40
PJ5P	9938	10571	10860	10456	472	46.08	49.57	49.96	48.54	2.14
PJ6P	11182	11727	11949	11619	394	55.53	51.77	52.29	53.20	2.03

Appendix 6 Results of chapter 4

Table 6.1 Changes in colour parameters of yoghurt (L*, a* and b*) by the addition of pomegranate juice concentrate (PLR: control and AC6: supplemented yoghurt samples)

Samples	L*			Ave	STD	a*			Ave	STD	b*			Ave	STD
	1	2	3			1	2	3			1	2	3		
PLR	92.58	92.80	92.62	92.67	0.12	-5.25	-5.14	-5.12	-5.17	0.07	12.77	12.66	12.53	12.65	0.12
AC6	74.59	75.25	75.14	74.99	0.35	5.26	6.99	7.11	1.04	6.45	5.26	5.40	5.17	5.28	0.12

Table 6.2 Changes in colour parameters of yoghurt (c, Hº and colour index) by the addition of pomegranate juice concentrate (PLR: control and AC6: supplemented yoghurt samples)

Samples	c			Ave	STD	Hº			Ave	STD	Colour index			Ave	STD
	1	2	3			1	2	3			1	2	3		
PLR	13.81	13.67	13.54	13.67	0.14	112.36	112.09	112.22	112.22	0.14	0.64	0.64	0.64	0.64	0.00
AC6	8.78	8.84	8.79	8.80	0.03	36.80	37.69	36.01	36.83	0.84	1.72	1.69	1.72	1.71	0.01

Table 6.3 Changes in textural properties of yoghurt as a result of pomegranate juice concentrate addition (PLR: control and AC6: supplemented yoghurt samples)

Samples	Fracture force (N)			Ave	STD	Firmness (N)			Ave	STD	Adhesiveness (N.s)			Ave	STD
	1	2	3			1	2	3			1	2	3		
PLR	1.51	1.48	1.48	1.49	0.02	1.80	1.67	1.86	1.78	0.10	6.16	6.02	6.19	6.13	0.09
AC6	1.25	1.31	1.26	1.27	0.03	1.77	1.66	1.58	1.67	0.09	4.91	5.00	5.01	4.97	0.05

Table 6.4 Variations in the viable counts of ST-B01, LA-5 and BB-12 in control yoghurt (PLR) during storage at 4 ℃

Cultures	Period (day)	PLR (log CFU/g)										
		First count						Second count			Average (total)	STD
		1	2	Average (1 & 2)	Repeatability limit, r = 0.25, in $\log_{10}$			1	2	Average (1 & 2)		
					Aver of first count	- 0.25 $\log_{10}$	+ 0.25 $\log_{10}$					
ST-B01	1	7.34	7.38	7.36	36	20	64	7.55	7.61	7.58	**7.47**	**0.16**
	7	7.27	7.29	7.28	28	16	50	7.43	7.46	7.45	**7.36**	**0.12**
	14	7.30	7.33	7.32	32	18	57	7.19	7.23	7.21	**7.26**	**0.07**
	21	7.49	7.54	7.52	52	29	93	7.30	7.33	7.32	**7.42**	**0.14**
	28	7.31	7.34	7.33	33	18	59	7.44	7.44	7.44	**7.38**	**0.08**
LA-5	1	6.60	6.62	6.61	61	34	109	6.82	6.85	6.84	**6.72**	**0.16**
	7	6.40	6.43	6.42	42	24	75	6.27	6.31	6.29	**6.35**	**0.09**
	14	6.30	6.33	6.32	32	18	57	6.52	6.55	6.54	**6.43**	**0.16**
	21	6.36	6.40	6.38	38	21	68	6.22	6.23	6.23	**6.30**	**0.11**
	28	6.18	6.22	6.20	20	11	36	6.32	6.35	6.34	**6.27**	**0.10**
BB-12	1	6.42	6.44	6.43	43	24	77	6.70	6.75	6.73	**6.58**	**0.21**
	7	6.36	6.40	6.38	38	21	68	6.56	6.62	6.59	**6.49**	**0.15**
	14	6.28	6.33	6.31	31	17	55	6.51	6.53	6.52	**6.41**	**0.15**
	21	6.38	6.38	6.38	38	21	68	6.23	6.28	6.26	**6.32**	**0.09**
	28	6.20	6.23	6.22	22	12	39	6.34	6.36	6.35	**6.28**	**0.10**

Table 6.5 Variations in the viable counts of ST-B01, LA-5 and BB-12 in PJC-supplemented yoghurt (AC6) during storage at 4 ℃

Cultures	Period (day)	AC6 (log CFU/g)										
		First count						Second count			Average (total)	STD
		1	2	Average (1 & 2)	Repeatability limit, r = 0.25, in $\log_{10}$			1	2	Average (1 & 2)		
					Aver of first count	- 0.25 $\log_{10}$	+ 0.25 $\log_{10}$					
ST-B01	1	7.08	7.13	7.11	111	62	198	7.27	7.30	7.29	**7.20**	**0.13**
	7	6.95	7.01	6.98	98	55	174	7.33	7.37	7.35	**7.17**	**0.26**
	14	7.07	7.12	7.10	110	62	196	7.31	7.32	7.32	**7.21**	**0.16**
	21	7.09	7.12	7.11	111	62	198	7.28	7.29	7.29	**7.20**	**0.13**
	28	6.67	6.71	6.69	69	39	123	6.88	6.93	6.91	**6.80**	**0.15**
LA-5	1	6.52	6.55	6.54	54	30	96	6.74	6.80	6.77	**6.65**	**0.17**
	7	6.27	6.27	6.27	27	15	48	6.42	6.49	6.46	**6.36**	**0.13**
	14	6.31	6.36	6.34	34	19	61	6.46	6.55	6.51	**6.42**	**0.12**
	21	6.07	6.10	6.09	109	61	194	6.28	6.33	6.31	**6.20**	**0.16**
	28	5.89	5.92	5.91	91	51	162	6.14	6.16	6.15	**6.03**	**0.17**
BB-12	1	6.58	6.59	6.59	59	33	105	6.32	6.37	6.35	**6.47**	**0.17**
	7	6.46	6.49	6.48	48	27	85	6.24	6.32	6.28	**6.38**	**0.14**
	14	6.36	6.36	6.36	36	20	64	6.19	6.24	6.22	**6.29**	**0.10**
	21	6.24	6.30	6.27	27	15	48	6.17	6.19	6.18	**6.23**	**0.06**
	28	5.96	6.01	5.99	99	55	176	6.08	6.18	6.13	**6.06**	**0.10**

Table 6.6 The viable counts of Lactic acid and probiotic bacteria in the control model system

Test system	Control ystem (log CFU/g)										
	First count						Second count			Average (total)	STD
	1	2	Average (1 & 2)	Repeatability limit, r = 0.25, in $\log_{10}$			1	2	Average (1 & 2)		
				Count	- 0.25 $\log_{10}$	+ 0.25 $\log_{10}$					
PLS	6.65	6.68	6.67	67	38	119	6.83	6.91	6.87	**6.77**	**0.14**
PLL	7.67	7.71	7.69	69	39	123	7.46	7.51	7.49	**7.59**	**0.14**
PLB	7.31	7.31	7.31	31	17	55	7.42	7.48	7.45	**7.38**	**0.10**

Table 6.7 The viable counts of Lactic acid and probiotic bacteria in the PJC-supplemented model system

Test system	Supplemented system (log CFU/g)										
	First count						Second count			Average (total)	STD
	1	2	Average (1 & 2)	Repeatability limit, r = 0.25, in $\log_{10}$			1	2	Average (1 & 2)		
				Count	- 0.25 $\log_{10}$	+ 0.25 $\log_{10}$					
POS	6.62	6.67	6.65	65	36	116	6.83	6.91	6.87	**6.76**	**0.16**
POL	7.62	7.73	7.68	68	38	121	7.49	7.54	7.52	**7.60**	**0.11**
POB	6.28	6.30	6.29	29	16	52	6.44	6.52	6.48	**6.39**	**0.13**

Table 6.8 Viable counts of lactic acid and probiotic bacteria in control freeze-dried yoghurt powder

Cultures	Control FDYP										
	First count						Second count			Average (total)	STD
	1	2	Average (1 & 2)	Repeatability limit, r = 0.25, in $\log_{10}$			1	2	Average (1 & 2)		
				Count	- 0.25 $\log_{10}$	+ 0.25 $\log_{10}$					
ST-B01	8.23	8.31	8.27	27	15	48	8.2	8.24	8.22	**8.25**	**0.04**
LA-5	7.45	7.52	7.49	49	27	87	7.29	7.35	7.32	**7.40**	**0.12**
BB-12	7.21	7.25	7.23	23	13	41	7.37	7.39	7.38	**7.31**	**0.11**

Table 6.9 Viable counts of lactic acid and probiotic bacteria in supplemented freeze-dried yoghurt powder

Cultures	Supplemented FDYP										
	First count						Second count			Average (total)	STD
	1	2	Average (1 & 2)	Repeatability limit, r = 0.25, in $\log_{10}$			1	2	Average (1 & 2)		
				Count	- 0.25 $\log_{10}$	+ 0.25 $\log_{10}$					
ST-B01	7.92	8.03	7.98	98	55	174	7.95	8.14	8.05	**8.01**	**0.05**
LA-5	7.31	7.38	7.35	35	20	62	7.23	7.25	7.24	**7.29**	**0.07**
BB-12	7.08	7.16	7.12	112	63	199	7.28	7.31	7.30	**7.21**	**0.12**