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# Probiotic and antioxidant properties of synbiotic yoghurt supplemented with Australian-grown 'Wonderful' pomegranate (*Punica granatum* L.) juice

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**Abstract.** Physicochemical properties of the Australian-grown 'Wonderful' pomegranate juice (WPJ) including total phenolic compounds (TPC) and antioxidant activity (AA) were determined and compared with four brands of imported pomegranate juices (IPJs). The TPC in WPJ was found to be  $2,400 \pm 200$  mg/l GAE while in the IPJs ranged from 1,000 to 2,800 mg/l GAE. The AA in WPJ was  $11.0 \pm 1.0$  mM/l TEAC and ranged from 5.5 to 14.5 mM/l TEAC in IPJs. A synbiotic yoghurt supplemented with different levels of IPJ was developed. Maximum single strength PJ supplementation level with no adverse effects on yoghurt attributes and probiotics viability was found to be 20% in heat-treated milk resulting in a TPC level of  $731 \pm 69$  mg/l GAE in synbiotic yoghurt.

**Keywords.** Pomegranate – Antioxidant activity – Total polyphenol content – Synbiotic yoghurt.

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## I – Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest edible fruits widely grown in many tropical and subtropical countries (Fadavi *et al.*, 2005). Over 1,000 cultivars of *Punica granatum* exist, originating from the Middle East, extending westward throughout the Mediterranean, and eastward to China and India, and onto the American South-West, California and Mexico (Cam *et al.*, 2009; Lansky and Newman, 2007). Pomegranate's use in Australia has been primarily as a backyard ornamental tree (Lye, 2008). While no formal statistics are available in Australia, it is estimated that nearly 250 ha are currently grown with a similar area projected for new plantation, with expected increase of over 1,000 ha in the next 5-10 years (Eccles, 2009).

Over the last two decades consumers have become more aware of the relationship between food intake and good health, especially from natural foods such as fruits and vegetables. Pomegranate fruit which is renowned for its health benefits has become very popular worldwide over the last few years (Eccles, 2009). According to Tezcan *et al.* (2009), clinical research studies suggest that pomegranate juice (PJ) can reduce the level of oxidized LDL (low-density lipoprotein) cholesterol, and increase the activity of serum high-density lipoprotein (HDL)-associated paraoxonase 1 (Aviram *et al.*, 2000 and 2004; Aviram and Dornfeld, 2001; Kaplan *et al.*, 2001). The PJ also helps keep the prostate specific antigen (PSA) levels stable in men and even slows its rise (Pantuck *et al.*, 2006), is 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

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**Abbreviations:** PJ, Pomegranate Juice; WPJ, Pomegranate juice from 'Wonderful' variety; LPJ, Juice extracted from large size 'Wonderful' pomegranates; SPJ, Juice extracted from small size 'Wonderful' pomegranates; IPJ, Imported pomegranate juice.

as prostate and colon cancer (Adams *et al.*, 2006; Adhami and Mukhtar, 2007; Khan *et al.*, 2007; Malik and Mukhtar, 2006; Seeram *et al.*, 2007). It is reported that PJ can improve the sperm quality (Turk *et al.*, 2008) and erectile dysfunction in male patients as well (Forest *et al.*, 2007).

Studies have shown that pomegranate is a good source of flavonoids (flavonols, flavanols and anthocyanins) and hydrolysable tannins (HTs) (Hernandez *et al.*, 1999; Martin *et al.*, 2009; Seeram *et al.*, 2006). Hydrolysable tannins consist of gallotannins (hydrolysed to gallic acid and glucose), and ellagitannins (hydrolysed to ellagic acid and glucose). Each ellagic acid consists of 2 gallic acids, so, the monomeric part of this phenolic fraction is gallic acid (Alighourchi *et al.*, 2008; Gil *et al.*, 2000; Gonzalez-Molina *et al.*, 2009; Kulkarni *et al.*, 2004, 2007; Seeram *et al.*, 2005; Tanaka *et al.*, 1985, 1986a, b). Chemical analyses have shown that HTs as the predominant type of polyphenols in PJ (Ben Nasr *et al.*, 1996; Gil *et al.*, 2000; Seeram *et al.*, 2006) are responsible for over 92% of its antioxidant activity (AA) (Seeram *et al.*, 2006), which is higher than red wine, green tea, cranberry, grapefruit and orange juices (3, 3, 2, 6 and 8 folds higher, respectively) (Azadzi *et al.*, 2005; Gil *et al.*, 2000; Rosenblat and Aviram, 2006; Tzulker *et al.*, 2007).

Due to increased consumer awareness of PJ antioxidant properties, the consumption of pomegranate has increased around the world. Currently pomegranate is mainly grown in the Northern Hemisphere. Australia has a diverse climate and there are areas that are very suitable for pomegranate production, which offers a counter-seasonal opportunity for Australian growers and exporters (Lye, 2008). There is an important body of work in the literature on the properties of pomegranate from different countries (Alighourchi *et al.*, 2008; Al-Said *et al.*, 2009; Fadavi *et al.*, 2005; Ozgen *et al.*, 2008; Pande and Akoh, 2009; Poyrazoglu *et al.*, 2002; Shwartz *et al.*, 2009; Tzulker *et al.*, 2007), but practically no published work on Australian PJ (Eccles, 2009). This study was therefore undertaken to characterise the juice extracted from the locally-grown pomegranate ('*Wonderful*' variety) and to evaluate the impact of processing on its TPC and AA levels, and to use the PJ in value-adding exercises to establish the maximum achievable level of supplementation in synbiotic yoghurt and its effect on probiotic activities.

## II – Materials and methods

### 1. Materials

*Pomegranate fruit and juice.* Two different sizes of fresh '*Wonderful*' pomegranates with average sizes of  $238 \pm 10$  and  $573 \pm 21$  g were selected for the study. The fruits were supplied by a grower in Robinvale (between Mildura and Swan Hill in the North-west of Victoria, Australia,  $34^{\circ} 35'$  S latitude and  $142^{\circ} 46'$  E longitude) during the harvest season in April, 2010. Four brands of imported PJs (IPJ) coded as TT, TB, UP, IT were purchased from the local market. Samples TT and IT were sold in tetra pack cartons; the TB was marketed in 1000 ml bottles and for UP was sold in 236 ml PET (polyethylene terephthalate) bottles. Labels on all products claimed 100% juice with no added ingredients.

*Chemicals and reagents.* All chemicals used were analytical grade and sourced from Sigma-Aldrich Pty. Ltd. (NSW, Australia). These included Gallic acid, Folin-Ciocalteu reagent (F-C), ABTS (2, 2' -azinobis-(3 ethylbenzothiazoline-6-sulphonic acid) diammonium salt), potassium persulphate and trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

*Yoghurt ingredients.* Homogenised and pasteurised low-fat milk (1.3% fat, REV, Pramilet, Australia) and low-heat skim milk powder (LHSMF, 0.9% milk fat, 96% total solids, Bola Foods Ltd, Melbourne, and Vic, Australia) were used to produce yoghurt. The freeze-dried (FD) probiotic culture selected for this project was ABT-5-Probio-Tec™ (Chr. Hansen Pty. Ltd., Bayswater, Vic. Australia), a mixture of *Lactobacillus acidophilus* (LA-5), *Bifidobacterium*

*bifidum* (BB-12) and *Streptococcus thermophilus* (ST). The culture was kept at -22 °C until required for yoghurt preparation as direct vat set (DVS).

## 2. Methods

**Extraction of pomegranate juice.** Arils of large and small pomegranate fruits were manually separated for juice extraction using an electrical juicer (Sunbeam, model IE-AD, Italy) in two stages (the pulp from first extraction stage was passed through the juicer again for further juice extraction). The fresh juices thus extracted from the large and small fruits (LPJ and SPJ) were pooled separately and stored in a blast freezer at -28 °C. The physicochemical and phytochemical properties of the thawed samples were determined and compared with those of IPJs. Six different processes were employed to extract PJ from small size pomegranates coded PJ1 to PJ6. PJs were extracted from manually separated arils either with an electrical juicer (PJ1) in two stages to improve yield (the pulp from the first stage was re-extracted) or by manually operated screw press (M-Press) (PJ2). For PJs 3 and 4 the outer leathery skin of pomegranates were peeled off, the fruit was then segmented and PJs were extracted from these segments (arils still inside the white pith) with electrical juicer either in single stage (PJ3), or in two stages followed by manual pressing of the residual pulp (PJ4). For PJs 5 and 6, chopped unpeeled fruits were processed in electrical juicer either in single stage (PJ5) or in two stages followed by manual pressing (PJ6). The fresh juices extracted from each step were pooled separately and used for yield calculation. Each stream was then divided into two lots, the first lot was frozen and stored in blast freezer at -28 °C (PJ1 – PJ6) and the second lot was heat treated as follows before storage at refrigerated temperature.

**Heat treatment of pomegranate juice.** Different streams of juices (coded PJ1P to PJ6P) were pasteurized at 90°C for 15 sec, immediately cooled in an ice bath to below 10°C aseptically transferred into 450 ml glass bottles and tightly sealed and stored at 4°C.

### *Determination of total phenolic compounds*

Total phenolic compounds were determined by Folin-Ciocalteu (FC) colorimetry method which is based on chemical reduction of a mixture of tungsten and molybdenum oxides (Singleton and Rossi, 1965). 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 were thawed first, then 20 µL of diluted (1:10 with Milli Q water) sample (PJ or yoghurt or gallic acid standard solution) was mixed with 1.58 ml Milli Q water in a 2-ml plastic cuvette. 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 °C). Then, 300 µL of 20% sodium carbonate solution was added to all cuvettes (except those containing yoghurt) and allowed to stand for 2 h at room temperature (RT) before reading the absorbance at 765 nm in a UV/VIS Spectrometer equipped with UV Winlab software (Lambda 35, Perkin Elmer, MA, USA). The mixture of yoghurt and reagents was centrifuged (after 90 min standing at RT) at 18,500 g for 30 min at 4 °C (5810R, Eppendorf Centrifuge, Hamburg, Germany), and the supernatant was used for absorbance reading as above. The results were expressed as mg gallic acid equivalent (GAE) in 1 L of sample by comparison with standard curve, which was obtained from different concentrations of gallic acid (50 to 1000 mg/l).

**Determination of antioxidant activity (AA).** Total antioxidant activity of PJ was measured spectrophotometrically based on the generation of ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic 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 *et al.*, 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<sup>•+</sup> chromophore was diluted with Milli Q water to an absorbance of 0.70 (±0.020) at 734 nm (Rice-Evans *et al.*, 1994; Robert *et al.*, 1999; Whitehead *et al.*, 1995; Zhou *et al.*, 2007). An

aliquot of 200  $\mu$ l of diluted PJ (1:50 with Milli Q water), or Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble synthetic vitamin E analogue calibration standard ranging from 50  $\mu$ M/l to 1 mM/l), or Milli Q water (blank) was mixed with 2.0 ml ABTS<sup>++</sup> in a plastic cuvette. The mixture was allowed to stand at RT for 10 min with continuous stirring before the absorbance was measured by UV/VIS spectrometer (Lambda 35, Perkin Elmer) at 734 nm. Absorbance values were taken to the standard curve prepared with synthetic antioxidant Trolox and results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) (Gill *et al.*, 2000). The TEAC is equal to the millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation (Antolovich *et al.*, 2002).

*Colour measurement.* Aliquots of 25 ml of experimental and commercial PJs were transferred into disposable plastic petri dishes, covered and the colour parameters were determined using a Chroma Meter CR-400 (Konica Minolta, Sensing, INC, Japan) according to Shwartz *et al.* (2009) and expressed in dimensions of L\*, a\*, b\*, C and H°. The mean values of triplicate readings were reported for each sample. Values of L\*<sup>-</sup> indicate darkness and L\*<sup>+</sup> indicate lightness of sample colour, while a\*<sup>-</sup> indicates green colour and a\*<sup>+</sup> indicates red colour. The b\*<sup>+</sup> indicates a more yellow colour and b\*<sup>-</sup> indicates blue colour. The chroma (C) value is calculated as  $C = (a^{*2} + b^{*2})^{1/2}$  and indicates the colour intensity or saturation. Hue angle H° is a parameter that is effective in evaluating visual colour appearance and is calculated as  $H^\circ = \tan^{-1}(b^*/a^*)$  (Solomon *et al.*, 2006). The colour index was calculated from  $(180 - H^\circ)/(L^* + C)$  (Shwartz *et al.*, 2009 & Tzulker *et al.*, 2007).

*Yoghurt preparation.* Plain yoghurt was made according to Paseephol *et al.* (2008). Briefly, the low-fat milk (2.1.3) was standardised with LHSMP to a total solid content of 16% and heat-treated at 90°C for 10 min, then cooled to 43°C and inoculated with freeze-dried ABT-5-Probio-Tec™ culture at a level 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°C. At pH 4.7 the samples were transferred to a cold room at 4 °C. The commercial IPJ sample IT was selected for preliminary supplementation trials to produce synbiotic yoghurt. Four supplementation levels of 9, 13, 17 and 20% were trialled before or after heat treatment while keeping solids content constant at 16%

*Physico-chemical analyses.* Total soluble solids of fresh PJ was determined according to AOAC (2002) refractive index method with a Shibuya hand-held refractometer (Japan) and reported as degree Brix (°B). Total solids of milk and yoghurt samples were determined using oven method according to Australian Standard (AS 2300.1.1-2008). pH values of all samples were measured using a pH-meter (HI 8424, Hanna instruments, USA). Titratable acidity (TA) of PJ samples was determined potentiometrically using 0.1M NaOH to the end point of pH 8.1 according to the AOAC (2000) and reported as % citric acid (g per 100 ml). pH and TA of yoghurt and milk samples were determined according to Australian Standards (AS 2300.1.6-2010 and AS 2300.2.10-2008).

*Statistical analyses.* All tests were conducted in triplicate and the mean values  $\pm$  standard deviation (SD) are reported. Statistical analyses were performed by applying one-way analysis 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).

### III – Results and discussion

#### 1. Physicochemical properties of pomegranate juices

The average weight of pomegranates was found to range between  $238 \pm 10$  g for small fruits and  $573 \pm 21$  g for the large ones. The yield of arils from small pomegranates was  $61.44 \pm$

2.11% but only  $45.58 \pm 2.71\%$  from the large fruits that appeared larger and more red than the small fruits arils. Juice yield from arils was comparable at  $74.18 \pm 3.19\%$  and  $76.54 \pm 2.38\%$  for small and large fruits respectively, however, on whole fruit basis the yield was expectedly lower, i.e.  $45.58 \pm 1.96\%$  from small fruits and  $34.89 \pm 1.15\%$  from larger fruits that had thicker skin.

Soluble solids in LPJ were significantly ( $P < 0.05$ ) higher ( $16.8 \pm 0.2$  °B) than that of SPJ ( $15.2 \pm 0.2$  °B). In comparison, only one of the IPJs showed high soluble solids content ( $16.1 \pm 0.1$  °B) while others were significantly ( $P < 0.05$ ) lower and ranged from 13.3 to 14.5 °B (Table 1). The SPJ was slightly more acidic than the LPJ ( $1.58 \pm 0.07\%$  vs.  $1.15 \pm 0.09\%$ ). 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 1).

**Table 1. Chemical properties, total phenolic compounds and antioxidant activity of pomegranate juice samples**

Samples	Soluble solids (°B)	pH	TA (% citric acid)	TPC GAE mg/l	AA (TEAC mM/l)
SPJ <sup>1</sup>	$15.2 \pm 0.2^{bc}$	$3.00 \pm 0.02^{de}$	$1.58 \pm 0.07^{ab}$	$2460 \pm 164^{ab}$	$11.06 \pm 0.91^{bc}$
LPJ <sup>2</sup>	$16.8 \pm 0.2^a$	$3.25 \pm 0.01^b$	$1.15 \pm 0.09^{cd}$	$2545 \pm 97^{ab}$	$11.36 \pm 0.94^{ab}$
IPJ (TT)	$14.1 \pm 0.1^{cd}$	$3.34 \pm 0.01^a$	$1.01 \pm 0.06^{de}$	$1923 \pm 177^{bc}$	$9.59 \pm 0.93^{bc}$
IPJ (TB)	$14.5 \pm 0.1^c$	$3.02 \pm 0.01^d$	$1.61 \pm 0.08^{ab}$	$1293 \pm 113^{cd}$	$6.91 \pm 0.97^{cd}$
IPJ (UP)	$16.1 \pm 0.1^{ab}$	$3.32 \pm 0.01^{ab}$	$1.09 \pm 0.05^{de}$	$2630 \pm 245^{ab}$	$13.43 \pm 0.99^{ab}$
IPJ (IT)	$13.3 \pm 0.1^d$	$3.15 \pm 0.01^c$	$1.27 \pm 0.07^{cd}$	$1193 \pm 171^d$	$6.48 \pm 0.90^{bc}$

<sup>1</sup>Juice produced from small size fruit's arils by electrical juicer in two stages; <sup>2</sup>Juice produced from large size fruit's arils by electrical juicer in two stages. IPJ: Imported pomegranate juices. The data represent the means  $\pm$  SD of triplicate analyses from each trial. ANOVA was used to determine the significance of differences at a confidence level of 0.05 as identified by different letters in the same column.

The colour evaluation results are presented in Table 2. The values of L\*, a\*, C, H and colour index were not significantly different ( $P < 0.05$ ) between SPJ and LPJ, however, SPJ showed higher b\* value (yellowness) than LPJ. Compared to the fresh juices (SPJ & LPJ), the colour values were significantly different ( $P < 0.05$ ) for commercial products (IPJs) that were all produced from concentrated PJ. The commercial products showed significantly higher ( $P < 0.05$ ) L\* values (i.e. brighter), a\* value (more red except for IT) and b\* value (yellow) 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 2). According to H° formula [ $H^\circ = \tan^{-1} (b^*/a^*)$ ] any increase in the redness of a sample (a\*+) or drop in its yellowness (b\*+) results in low H° value. The H° values of IPJs were significantly higher ( $P < 0.05$ ) than SPJ and LPJ, while their colour index values were significantly lower ( $P < 0.05$ ) than SPJ and LPJ (Table 2). These results were correlated with visual appearances of samples and indicated that SPJ and LPJ's colour were more appealing and the commercial products showed inferior colour compared to fresh juices (Shwartz *et al.*, 2009; Tzulker *et al.*, 2007).

## 2. Effects of extraction method on pomegranate juice properties

The yields of PJ1, 2, 3 and 5 were not significantly ( $P < 0.05$ ) different. Considering the yields of PJ1 and 2 (produced from same raw materials), double extraction with electrical juicer and manual pressing methods had the same effects on the final yields. But when these two methods were used in combination, it increased the yield of final product significantly ( $P < 0.05$ ). Thus, PJ4 had the highest yield followed by PJ6 (Table 3).

**Table 2. Colour evaluation of pomegranate juice samples**

Samples	L*	a*	b*	c	H°	Colour index
SPJ <sup>1</sup>	17.50 ± 0.08 <sup>e</sup>	15.83 ± 0.70 <sup>cd</sup>	7.89 ± 0.46 <sup>d</sup>	17.69 ± 0.83 <sup>de</sup>	26.50 ± 0.36 <sup>e</sup>	4.36 ± 0.10 <sup>b</sup>
LPJ <sup>2</sup>	17.45 ± 0.32 <sup>e</sup>	15.43 ± 1.50 <sup>cd</sup>	6.24 ± 0.58 <sup>ed</sup>	16.64 ± 1.60 <sup>de</sup>	22.05 ± 0.44 <sup>e</sup>	4.64 ± 0.25 <sup>b</sup>
IPJ (TT)	26.67 ± 0.18 <sup>d</sup>	20.38 ± 0.96 <sup>bc</sup>	21.33 ± 0.80 <sup>ab</sup>	29.36 ± 1.48 <sup>bc</sup>	46.32 ± 0.30 <sup>c</sup>	2.39 ± 0.07 <sup>d</sup>
IPJ (TB)	27.16 ± 0.55 <sup>dc</sup>	23.22 ± 2.06 <sup>b</sup>	17.86 ± 1.90 <sup>b</sup>	29.29 ± 2.79 <sup>bc</sup>	37.54 ± 0.54 <sup>d</sup>	2.53 ± 0.16 <sup>d</sup>
IPJ (UP)	25.37 ± 0.99 <sup>d</sup>	29.07 ± 1.22 <sup>ab</sup>	19.56 ± 1.20 <sup>ab</sup>	35.04 ± 1.68 <sup>ab</sup>	33.92 ± 0.52 <sup>d</sup>	2.42 ± 0.10 <sup>d</sup>
IPJ (IT)	43.18 ± 0.89 <sup>a</sup>	6.67 ± 0.02 <sup>ed</sup>	23.46 ± 0.74 <sup>a</sup>	24.40 ± 0.72 <sup>cd</sup>	74.11 ± 0.42 <sup>ab</sup>	1.57 ± 0.04 <sup>e</sup>

<sup>1</sup>Juice produced from small size fruit's arils by electrical juicer in two stages; <sup>2</sup>Juice produced from large size fruit's arils by electrical juicer in two stages. IPJ: Imported pomegranate juices. The data represent the means ± SD of triplicate analyses from each trial. ANOVA was used to determine the significance of differences at a confidence level of 0.05 as identified by different letters in the same column.

**Table 3. Yield and chemical properties of pomegranate juices extracted with different methods and the effect of heat treatment on TPC and AA of these juice sample**

Samples	Yield <sup>1</sup>	Soluble solids (° B)	pH	TA (% citric acid)
PJ1	45.58 ± 1.96 <sup>bc</sup>	15.2 ± 0.2 <sup>d</sup>	3.00 ± 0.02 <sup>de</sup>	1.58 ± 0.07 <sup>abc</sup>
PJ2	45.04 ± 2.06 <sup>bc</sup>	15.2 ± 0.2 <sup>d</sup>	3.00 ± 0.01 <sup>de</sup>	1.64 ± 0.10 <sup>ab</sup>
PJ3	45.23 ± 2.07 <sup>bc</sup>	15.9 ± 0.1 <sup>c</sup>	3.08 ± 0.01 <sup>bc</sup>	1.32 ± 0.07 <sup>cd</sup>
PJ4	56.87 ± 1.64 <sup>a</sup>	16.1 ± 0.1 <sup>bc</sup>	3.10 ± 0.01 <sup>b</sup>	1.42 ± 0.09 <sup>bcd</sup>
PJ5	40.04 ± 2.04 <sup>bc</sup>	16.4 ± 0.2 <sup>bc</sup>	3.10 ± 0.01 <sup>ab</sup>	1.36 ± 0.07 <sup>cd</sup>
PJ6	49.78 ± 2.12 <sup>cd</sup>	16.9 ± 0.1 <sup>ab</sup>	3.10 ± 0.02 <sup>b</sup>	1.38 ± 0.09 <sup>cd</sup>

Samples	TPC (GAE mg/l)		AA (TEAC mM/l)	
	BHT <sup>2</sup>	AHT <sup>3</sup>	BHT <sup>2</sup>	AHT <sup>3</sup>
PJ1	2460 ± 164 <sup>de</sup>	2293 ± 248 <sup>e</sup>	11.06 ± 0.91 <sup>d</sup>	10.24 ± 0.81 <sup>d</sup>
PJ2	2071 ± 62 <sup>e</sup>	2064 ± 123 <sup>e</sup>	9.16 ± 0.97 <sup>d</sup>	9.03 ± 0.95 <sup>d</sup>
PJ3	5760 ± 609 <sup>cd</sup>	5297 ± 746 <sup>d</sup>	23.13 ± 2.88 <sup>c</sup>	21.36 ± 2.63 <sup>c</sup>
PJ4	7293 ± 605 <sup>c</sup>	6930 ± 555 <sup>c</sup>	30.25 ± 2.10 <sup>bc</sup>	27.70 ± 1.40 <sup>bc</sup>
PJ5	11545 ± 503 <sup>ab</sup>	10456 ± 472 <sup>b</sup>	50.65 ± 1.60 <sup>a</sup>	48.54 ± 2.14 <sup>a</sup>
PJ6	12516 ± 167 <sup>ab</sup>	11619 ± 394 <sup>ab</sup>	56.91 ± 2.79 <sup>a</sup>	53.20 ± 2.03 <sup>a</sup>

<sup>1</sup>Yield calculated on whole fruit base; <sup>2</sup>Before heat treatment; <sup>3</sup>After heat treatment (90 °C for 15 sec). Data shown represents the means ± SD of triplicate analyses from each trial. ANOVA was used to determine the significance of differences at a confidence level of 0.05 as identified by different letters in the same column.

Raw materials and extraction methods affected soluble solids content of each sample. Accordingly, soluble solids in PJ1 and 2 (15.2 ± 0.2) produced from arils with the same yields and extraction methods were not significantly ( $P < 0.05$ ) different.

Based on raw materials used and extraction method employed, soluble solids in PJ5 was significantly ( $P < 0.05$ ) higher (16.4 ± 0.2) than that of PJ3 (15.9 ± 0.1), and PJ6 showed significantly ( $P < 0.05$ ) higher soluble solids (16.9 ± 0.1) than PJ4 (16.1 ± 0.1), while all other samples showed similar soluble solids content. The higher soluble solids content in PJ4 and 6 resulted from the extraction methods used (Table 3).

The titratable acidity of PJ1 (1.58 ± 0.07) was close ( $P < 0.05$ ) to PJ2 (1.64 ± 0.10) and both were slightly more acidic than other samples that were not significantly different ( $P < 0.05$ ) (Table 3) in acidity.

Difference in raw materials and extraction methods affected the colour parameters of extracted juices. The correlations between these parameters are reported in Table 4. No significant

differences ( $P < 0.05$ ) were found between the  $L^*$  values of PJ1 and 5, PJ2 and 3, and PJ4 and 6; between the  $a^*$  values of PJ1 and 3, PJ5 and 6; and between the  $b^*$  values of PJ4 and 5. PJ1 showed the highest  $H^\circ$  value ( $26.50 \pm 0.36$ ) that declined as the extraction method became more extensive, the lowest value found in PJ6 ( $14.13 \pm 0.96$ ). The colour index was not affected by the type of raw materials used, and in contrast with  $H^\circ$  values, the highest and lowest colour index was observed in PJ6 ( $4.68 \pm 0.19$ ) and PJ1 ( $4.36 \pm 0.10$ ) respectively.

**Table 4. Colour evaluation of pomegranate juices extracted with different methods**

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

The data represent the means  $\pm$  SD of triplicate analyses from each trial. ANOVA was used to determine the significance of differences at a confidence level of 0.05 as identified by different letters in the same column.

### 3. Phytochemical content of pomegranate juice

The health benefits attributed to pomegranate fruit consumption are related, at least in part, to their antioxidant activity (AA) (Shwartz *et al.*, 2009; Vardin & Fenercioglu, 2003). The AA of SPJ, LPJ and IPJs were determined using ABTS method (section 2.2.3) and the results were expressed as mM/I TEAC. The AA of SPJ and LPJ were 11.06 and 11.36 mM/I TEAC, while IPJs (coded UP, TT, TB and IT) showed 13.43, 9.59, 6.91 and 6.48 mM/I TEAC, respectively (Table 1).

In pomegranates like many other fruits and vegetables, the level of AA can be attributed to the level of TPC (Gil *et al.*, 2000; Shwartz *et al.*, 2009; Solomon *et al.*, 2006; Tzulker *et al.*, 2007). Therefore, TPC in these juices were also measured by F C method (section 2.2.2) and the results were expressed as mg/l GAE (Table 1).

Freshly extracted juices SPJ and LPJ contained 2,460 and 2,545 mg/l GAE, respectively; while the IPJs (UP, TT, TB and IT) contained 2,630, 1,923, 1,293 and 1,193 mg/l GAE, respectively (Table 1). Gil *et al.* (2000) suggested that the industrial extraction process either increases the amount or enhances the activity of the antioxidants. Industrially, the whole fruit is pressed hydrostatically which results in the extraction of a large amounts of polyphenols from the peels (Tzulker *et al.*, 2007). The juice is then filtered, concentrated, stored and sold to juice packaging companies where it is diluted with water and packed. It is this level of dilution that determines the TPC and the AA of the juice. Among the IPJs tested in this project, only sample coded UP showed a comparable TPC level to fresh juice obtained from arils while other samples failed in this regard. These results are in agreement with the TSS of samples tested although TSS alone could not be a good guide to AA activity, since some IPJs may have added sugar to adjust their TSS to an acceptable level.

These results confirm that the AA of different samples was directly related to the levels of TPC in those samples. Thus, any process that increases the level of TPC in a sample will result in an increased antioxidant activity.



#### 4. Effects of fruit parts and extraction procedures on phytochemical content of pomegranate juice

To improve the TPC level in PJ samples different parts of pomegranate fruits were used and combined with different extraction procedures (PJ1 to PJ6). In juices extracted from arils, the TPC level in PJ1 ( $2460 \pm 164$  mg/l GAE) was marginally higher than PJ2 ( $2071 \pm 62$  mg/l GAE) due to the extraction method employed but the AA of both samples were not statistically different ( $P < 0.05$ ) (Table 2). The TPC level and AA of PJs from peeled fruits were higher than those from arils. On the other hand, due to intensity of extraction method PJ4 showed significantly ( $P < 0.05$ ) higher TPC and AA ( $7293 \pm 605$  mg/l GAE and  $30.25 \pm 2.10$  mM/l TEAC) than PJ3 ( $5760 \pm 609$  mg/l GAE and  $23.13 \pm 2.88$  mM/l TEAC) (Table 2).

Upon using unpeeled whole fruits for juice extraction the TPC level and AA of PJs 5 and 6 were further increased compared to all other samples (Table 2), however, changing the extraction method from PJ5 to PJ6 did not significantly ( $P < 0.05$ ) affect the TPC content ( $11545 \pm 503$  vs.  $12516 \pm 167$  mg/l GAE) or the antioxidant activity ( $50.65 \pm 1.60$  vs.  $56.91 \pm 2.79$  mM/l TEAC). The TPC level in PJ1P, 3P and 5P were marginally lower than PJ1, 3 and 5 respectively, while in other samples the TPC level remained unchanged ( $P < 0.05$ ) after heat treatment ( $90^\circ\text{C}$  for 15 sec) (Table 2). Results obtained revealed that by changing the extraction methods the TPC level in PJ4 and 6 increased, however, heat treatment did not significantly ( $P < 0.05$ ) affect the TPC levels in these samples (PJ4P and 6P). Similarly, heat treatment did not adversely affect the AA of all samples (Table 2).

#### 5. Symbiotic yoghurt supplemented with pomegranate juice

A synbiotic product was developed by incorporating PJ in the formulation of probiotic yoghurt. The IPJ coded IT was used in the preliminary supplementation trials. Different supplementation levels (9, 13, 17 and 20%) were trialled before and after heat treatment of milk at  $90^\circ\text{C}$  for 10 min. Supplementation with PJ before heat treatment was limited to 9%, beyond which the milk curdled, whereas after heat treatment up to 20% PJ could be added without any adverse effect. It appears that heat treatment of standardised milk increased its stability and the buffering capacity of milk proteins. The supplemented milk samples were inoculated with probiotic culture and incubated at  $43^\circ\text{C}$ . The activity of the cultures was monitored by measuring the pH (Fig. 1) and enumeration technique. Upon reaching pH 4.7 the incubation time was recorded and yoghurt samples were transferred to refrigerated storage at  $4^\circ\text{C}$ . Comparing the yoghurt setting times it was noted that PJ supplementation up to 20% did not adversely affect the cultures activity or yoghurt attributes.

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

The number of ST-B01 in control sample decreased marginally in day 7 but did not significantly ( $P < 0.05$ ) change during the subsequent storage period. In the PJ-supplemented sample the population of ST-B01 declined by ca. 0.2 log cycles in day 1 and remained stable for the next 3 weeks, and then dropped by ca. 0.4 log cycles in the last week of the 4-week storage period, resulting in a viability of 98.8% in the control and 94.4% in the PJ-supplemented sample. The initial count of LA-5 ( $6.72$  log CFU/g in control and  $6.65$  log CFU/g in supplemented sample) demonstrated a steady but slow decline reflecting a small effect of the PJ supplementation. By the end of storage, the population of LA-5 in control yoghurt dropped to  $6.27$  log CFU/g, i.e. a viability of 93%, while the PJ-supplemented yoghurt showed  $6.03$  log CFU/g i.e. 90% viability. A steady decline in the numbers of BB-12 was also observed at the end of the storage period, from the initial counts of  $6.58$  log CFU/g to  $6.28$  log CFU/g in control and from  $6.47$  log CFU/g to  $6.06$  log CFU/g in PJ-supplemented sample, corresponding to a viability of 95.5% and 93.7% respectively.

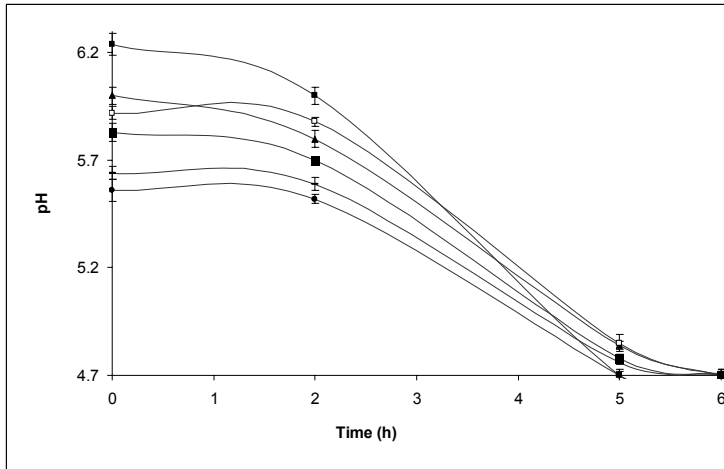


Fig. 1. pH range in yoghurts supplemented with P.J. • Plain yoghurt; ■ +13% PJ after heat treatment; ▲ +9% PJ before heat treatment; — +17% PJ after heat treatment; □ +9% PJ after heat treatment; ● +20% PJ after heat treatment.

Table 5. Variations in the viable counts of ST-B01, LA-5 and BB-12 in control and Synbiotic (PJC-supplemented) yoghurts during storage at 4°C

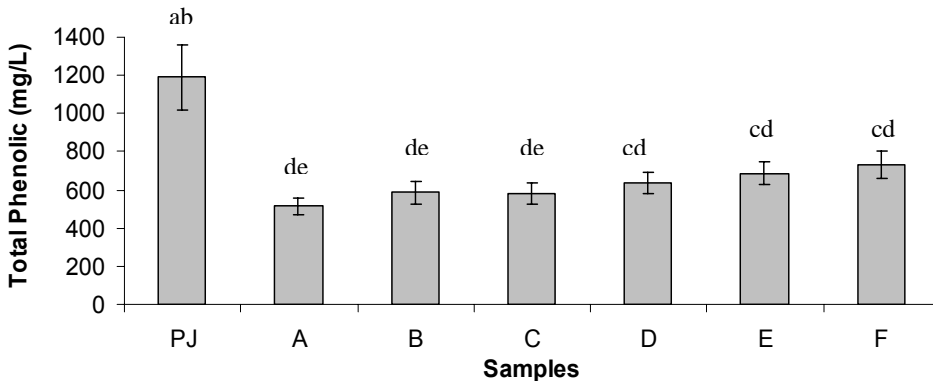
Culture	Period (day)	Control yoghurt (log CFU/g)	Synbiotic yoghurt (log CFU/g)
ST-B01	1	7.47 ± 0.16 <sup>abc</sup>	7.20 ± 0.13 <sup>ab</sup>
	7	7.36 ± 0.12 <sup>bcd</sup>	7.17 ± 0.26 <sup>ab</sup>
	14	7.26 ± 0.07 <sup>cde</sup>	7.21 ± 0.16 <sup>ab</sup>
	21	7.42 ± 0.14 <sup>bcd</sup>	7.20 ± 0.13 <sup>ab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

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.

These observations were 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.

The TPC content of synbiotic yoghurts containing 9, 13, 17 and 20% PJ was found to be 583, 637, 688 and 731 mg/l GAE, respectively against a background TPC in plain yoghurt (control) of 514 mg/l GAE (Fig. 2).



**Fig. 2.** Total phenolic compounds (mg/l GAE) in IPJ (coded IT) and in yoghurts supplemented with IPJ (IT). The data represent the means  $\pm$  SD of triplicate analyses from each trial. ANOVA was used to determine the significance of differences at a confidence level of 0.05 as identified by different letters. A: Plain yoghurt; B: +9% PJ before heat treatment; C: +9% PJ after heat treatment; D: +13% PJ after heat treatment; E: +17% PJ after heat treatment; F: +20% PJ after heat treatment.

## IV – Conclusions

The physicochemical and phytochemical properties of the Australian 'Wonderful' pomegranates grown in Robinvale (Victoria) were determined and compared with 4 different brands of imported commercial pomegranate juices. The larger fruits seemed to have lower yield of juice, that was slightly less sour and sweeter (higher TSS), and contained over 3.3% more TPC and nearly 2.7% higher AA than juice from smaller fruits. Freshly extracted Juices showed higher TPC and AA than three of imported commercial PJs except the sample coded UP which had comparable levels of TPC and AA.

Up to six fold increase in TPC level could be achieved in PJs using different extraction methods and various parts of the fruit. While, strong astringency of these juices could limit their applications for direct or combined consumption, they may however find uses in nutraceuticals as AA supplements. Pasteurisation (90°C for 15 sec) did not significantly ( $P < 0.05$ ) affect the AA of the extracted PJs, although TPC levels of PJ1, PJ3 and PJ5 slightly declined after heat treatment.

In the production of synbiotic yoghurt up to 20% single strength PJ could be added to heat-treated milk, and no apparent antagonism was observed between the cultures viability and the PJ phytochemicals. Considering the recommended polyphenols intake of ca. 1 g/day (Baghurst, 2006) this synbiotic yoghurt offers a pleasant and effective route to increasing the antioxidant intake in our daily diet.

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